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THE INFLUENCE OF PLANE OF NUTRITION AND OF ENVIRONMENTAL TEMPERATURE ON THE RELATIONSHIP BETWEEN BASAL METABOLISM AND ENDOGENOUS NITROGEN METABOLISM SUBSEQUENTLY DETERMINED [1]

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(Received for publication June 23, 1941)

The demonstration by Terroine and Sorg-Matter ('27), amply confirmed by Smuts ('35) and by Brody, Procter and Ashworth ('34), that there is a relationship approaching constancy between the endogenous nitrogen metabolism and the basal metabolism in adult animals maintained under comparable conditions imposing no strain upon the heat regulating mechanism, is a most important scientific contribution with important practical applications. Since the endogenous loss of nitrogen is the predominant component among the factors determining the maintenance requirement of protein, the constancy of this ratio among adult animals means that the maintenance requirement of protein varies with body size in a manner similar to the basal metabolism, i.e., in proportion to body surface, not in proportion to body weight as is all too generally presumed.

The importance of this relationship justifies an extension of its study to immature animals and to animals under variable environmental conditions. This paper reports the results of experiments on rats on the effect of variable caloric intake

[1] The data reported in this paper were taken from a thesis presented by Ray Treichler to the Graduate School of the University of Illinois in 1939 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Animal Husbandry.

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and of low environmental temperature on the basal metabolism and the endogenous nitrogen metabolism and on the ratio of the two. These variable conditions were imposed for a period of time prior to the measurements themselves, which were carried out according to the usual standardized procedures. The effect of variable age will be reported later.

The imposition of low environmental temperature prior to the basal metabolism determination has been shown to exert a stimulating effect, direct or indirect, on the basal metabolism of albino rats by Benedict and MacLeod ('29), Giaga and Gelineo ('30), Ring ('36, '39), Schwabe and Griffith ('36), Schwabe, Emery and Griffith ('38), and Collip and Billingsley ('36, '37). A stimulating effect of low temperature on the endogenous nitrogen excretion has been reported by Terroine and Sorg-Matter ('28) on mice and by Fontaine, Guillemet and Mandel ('38) on dogs. But whether the two effects are proportional so that the constancy of the ratio of endogenous nitrogen to basal heat is maintained, awaits simultaneous measurements on the same animals.

The plane of nutrition has also been found to exert an influence on the basal metabolism of the albino rat. Particularly evident has been the depressing effect of undernutrition (Gulick, '22, '24; Aszodi, '24; Horst, Mendel and Benedict, '34). With other species of animals, also, including man, undernutrition has generally been found to be associated with a subnormal metabolic rate. However, a stimulating effect of overnutrition has not been generally observed. For the growing albino rat, Hamilton ('37) has shown conclusively that there is a direct and close relationship between variations in the previous caloric intake and variations in the basal metabolism. No evidence seems to be at hand on the effect of plane of nutrition on the endogenous nitrogen metabolism.

PLAN OF THE EXPERIMENT

The investigation involved a study of the effect of varying two factors, (a) the plane of nutrition, and (b) the environmental temperature, upon subsequent determinations of basal

metabolism and endogenous nitrogen excretion in the urine carried out according to standard procedures.

Except for those animals subjected to subcritical temperatures, all experimental animals were maintained at an environmental temperature of approximately 28°C. Basal metabolism and endogenous nitrogen excretion were measured at, or slightly above, 28°C. In the test on the effects of low environmental temperature, the animals were maintained at approximately 4°C. for a minimum of 1 week prior to the experimental measurements. The level of food intake was maintained constant as closely as possible throughout the entire experimental period. The experimental diets are described in table 1.

TABLE 1

Percentage composition of experimental diets.

INGREDIENTS	DIET 293 10% PROTEIN	DIET 292 4% PROTEIN	DIET 296 PROTEIN-FREE
Dried ether-extracted whole egg	14.69	5.82	....
Celluflour [1]	4.00	4.00	4.00
Cornstarch	48.31	57.18	57.18
Sucrose	10.00	10.00	15.82
Wesson salts [2]	5.00	5.00	5.00
Corn oil	8.00	8.00	8.00
Butterfat	8.00	8.00	8.00
Cod liver oil	1.50	1.50	1.50
Wheat germ oil	0.50	0.50	0.50
Per cent nitrogen [3]	1.68	0.69	0.04
Calories per gram [3]	4.80	4.65	4.57

[1] Product of the Chicago Dietetic Supply House. Contains 37.8% crude fiber and 0.015% nitrogen.

[2] Wesson ('32) slightly modified to contain traces of cobalt and zinc.

[3] These values are averages of all the diets made up during the course of the experiment.

In the first experiment, sixteen mature male albino rats were selected. They were separated into two groups of eight each, and so fed that at the time of the basal energy determinations they approximated each other in weight and age. The one group (rats 37–44, inclusive) was fed diet 293 for mainte-

nance of weight only, while the other group (rats 45–52, inclusive) received the same diet at a higher caloric intake, which permitted a slow gain.

In the second experiment, which involved variation of both caloric intake and environmental temperature, fifteen male albino rats were used. They were separated into five trios, the rats in each trio approximating each other in age and weight. One rat in each trio was fed diet 293 at a level required for maintenance of body weight and at an environmental temperature of approximately 4°C. The second rat in each trio received the same amount of the same diet, or as close to the same amount as it was possible to get it to eat, at an environmental temperature of 28°C. The third rat in each trio was fed maintenance amounts of diet 293 at an environmental temperature of 28°C.[2]

In determining the endogenous level of nitrogen excretion in the urine, collections were obtained both on the 4% egg-protein diet (no. 292) and on the protein-free diet (no. 296) with quite comparable results. Ferric oxide was used in all cases to separate the fecal collections.

Basal energy determinations were made by the gravimetric method of Haldane (1892) as modified by Mitchell and Carman ('26). Those rats maintained at an environmental temperature of 4°C. were removed to an environmental temperature of 28°C. 4 hours prior to the start of the basal energy measurement. Immediately preceding and following basal energy measurements, the body temperature was taken, using a rectal clinical thermometer inserted ad rectum. Body temperature was taken over a 7-week period on those rats used in the environmental temperature and food intake study. The details of the various technical procedures used in this experiment were the same as those described by Smuts ('35).

EXPERIMENTAL RESULTS

The results of the first experiment are summarized in table 2. The average basal metabolic rate of the rats on the higher

[2] The relative humidities at the two experimental temperatures were 76% in the cold room and 32% in the warm room.

TABLE 2

Summary of results on rats receiving different caloric intakes. Experiment I.

	RATS 37–44	RATS 45–52	SIGNIFICANCE OF MEAN DIFFERENCE [2]		
			s	t	Odds
Weight, gm.	231	252			
Food intake, cal.	37.51	45.34			
Basal heat, cal./m²/day [1]	707	766	47.74	2.44	62:1
Endogenous N, mg./m²/day [1]	1535	1472	64.37	1.98	27:1
Total creatinine N, pct. of endogenous N, pct.	8.00	7.79	0.45	0.9	4.7:1
Ratio of endogenous N to basal heat, mg./cal.	2.18	1.85	0.23	2.64	>100:1

[1] Body surfaces were estimated from body weight by the formula of Lee ('29):

$$S_{cm^2} = 1254\ W_{gms.}^{.6}$$

[2] R. A. Fisher: Statistical Methods for Research Workers. London. 1928. 2nd ed. P. 107.

plane of nutrition was 8.3% higher than that of the rats on the lower plane of nutrition and the difference was highly significant statistically. That it was a result of the higher plane of nutrition rather than of a greater growth rate may be inferred from the experiments of Hamilton ('37), which showed that different rates of growth unassociated with different caloric intakes do not induce variations in the basal metabolic rate in rats.

The endogenous nitrogen output in the urine per square meter of body surface averaged about 4% less on the higher plane of nutrition, though the odds favoring the significance of this difference are not sufficiently great to permit a very positive conclusion. The proportion of total creatinine nitrogen to total endogenous nitrogen was obviously not affected by the plane of nutrition.

As a consequence of the effects of the plane of nutrition on the basal metabolic rate on the one hand, and on the endogenous nitrogen output on the other, the ratio of endogenous nitrogen to basal calories was considerably and significantly depressed by the higher caloric intake, the average ratios being 2.18 on the lower plane of nutrition and 1.85 on the higher.

TABLE 3

Summary of data obtained in studies involving different environmental temperatures and food intake levels.

TRIO	GROUP NO.[1]	RAT NO.	INITIAL WEIGHT	AVERAGE DAILY CALORIC INTAKE		BASAL HEAT	ENDOGENOUS NITROGEN	TOTAL CREATININE N PER CENT OF ENDOGENOUS N	RATIO ENDOGENOUS N TO BASAL HEAT
				Prior to basal	During collection				
						cal./m²/day	*mg./m²/day*	%	*mg./cal.*
1	1	53	250	78	83	876	1728	5.90	1.97
	2	54	256	68	50	871	1474	8.55	1.69
	3	55	241	39	40	736	1558	8.27	2.12
2	1	56	214	75	78	760	1946	5.88	2.56
	2	57	213	59	78	763	1356	8.46	1.78
	3	58	205	35	32	689	1386	7.88	2.01
3	1	59	260	78	83	863	1840	5.24	2.13
	2	60	266	71	54	846	1210	9.98	1.43
	3	61	263	37	32	636	1280	8.81	2.01
4	1	62	233	77	83	819	1493	6.17	1.82
	2	63	243	71	52	900	1526	5.96	1.70
	3	64	250	35	37	619	1155	9.47	1.87
5	1	65	237	79	82	809	1573	6.29	1.94
	2	66	238	64	41	821	1411	7.12	1.72
	3	67	238	35	37	712	1229	9.22	1.73
Aver.	1		239	77	82	825	1716	5.90	2.08
	2		243	67	55	840	1395	8.01	1.66
	3		239	36	36	678	1322	8.73	1.95

[1] 1 = 4°C., maintenance; 2 = 28°C., excess food; 3 = 28°C., maintenance.

The data obtained in the second experiment[3] are summarized in table 3, while the results of the statistical treatment of the permissible comparisons are presented in table 4.

The first comparison in table 4 shows that the basal metabolic rate is significantly increased by prior exposure of the rat to a low environmental temperature, the food intake being adjusted at both temperatures to maintain a constant body

[3] The mean body temperatures for the various groups of rats in the second experiment were as follows: 4°C. maintenance 38.3 ± .06°C.; 28°C. maintenance 38.5 ± .06°C.; and 28°C. supermaintenance 38.2 ± .08°C. No effect of prior environmental temperature or of plane of nutrition is revealed by these measurements. Furthermore, no significant correlation was found to exist between body temperature and basal metabolic rate.

weight. In the second comparison, feeding the rat at a supermaintenance level of nutrition, environmental temperature remaining constant, is associated with a highly significant increase in the basal metabolic rate, substantiating the findings presented in table 1. In the third comparison no significant difference could be found in the basal metabolic rate of rats exposed to temperatures of 4°C. and 28°C. when no great difference in the intake of food was simultaneously imposed. Inexplicably, the rats previously exposed to a temperature of 4°C., exhibited a significantly lower percentage of creatinine nitrogen in the endogenous urinary nitrogen, averaging 2 to 3% lower.

Thus, the basal metabolic rate was not elevated by prior exposure to a low environmental temperature unless the intake of food was increased at the low temperature in order to cover the increased energy requirements. Also, for the two groups of rats at 28°C., an average elevation in caloric intake somewhat less than that obtaining between the maintenance rats at 4°C. and at 28°C., occasioned an increase in basal metabolic rate of the same order of magnitude. The picture presented is thus consistent with the view that the effective agent in the stimulation of basal metabolism is the prior caloric intake, not the prior exposure to a low temperature.

Previously reported experiments purporting to show that exposure to low temperatures raises the basal metabolic rate in a subsequent period are probably subject to the same interpretation, since food intakes at the low temperatures employed would naturally be greater than food intakes at higher temperatures.

The excretion of endogenous nitrogen behaves differently. Here, a considerable difference in caloric intake at 28°C. (comparison II) was associated with no significant difference [4]

[4] The average effect of a difference in the plane of nutrition on the excretion of endogenous nitrogen in the second experiment (comparison II, table 4) is diametrically opposed to that observed in the first experiment (table 2), indicating the insignificance of the latter, although the point probably needs further study. The comparatively low odds obtained in comparison III are assumed to be fortified by the high odds in comparison I and the obviously insignificant odds in comparison II.

TABLE 4

Results of statistical analysis of the observed differences at different environmental temperatures and diet intake levels.

GROUP COMPARISONS	MEAN OF DIFFERENCES[1]	t	ODDS
I			
4°C. maintenance vs. 28°C. maintenance			
Basal heat production, cal. per m^2 per day	—147.0	4.96	>100 to 1
Endogenous nitrogen excretion, mg. per m^2 per day	—371.0	5.74	>100 to 1
Total creatinine N, per cent of endogenous N, pct.	2.83	9.79	>100 to 1
Ratio of endogenous N to basal heat, mg. per cal.	—0.136	1.12	4 to 1
II			
28°C. excess food vs. 28°C. maintenance			
Basal heat production, cal. per m^2 per day	—161.8	4.34	>100 to 1
Endogenous nitrogen excretion, mg. per m^2 per day	—73.8	0.83	3 to 1
Total creatinine N, per cent of endogenous N, pct.	0.716	0.80	3 to 1
Ratio of endogenous N to basal heat, mg. per cal.	0.284	2.84	41 to 1
III			
4°C. maintenance vs. 28°C. excess food			
Basal heat production, cal. per m^2 per day	14.8	0.86	4 to 1
Endogenous nitrogen excretion, mg. per m^2 per day	—320.6	2.52	28 to 1
Total creatinine N, per cent of endogenous N, pct.	2.12	2.49	27 to 1
Ratio of endogenous N to basal heat, mg. per cal.	—0.42	3.13	49 to 1

[1] Student: Biometrika, vol. 6, p. 1, 1908. +, 1st group lower than 2nd group; —, 1st group higher than 2nd group.

in the endogenous output of nitrogen, while a difference in prior environmental temperature, whether accompanied by a large (comparison I) or by a small (comparison III) difference in caloric intake, was associated with a significant difference in the excretion of endogenous nitrogen, the greater excretion occurring at the lower temperature. The effective agent here seems to be prior environmental temperature, not prior plane of nutrition.

The constant relationship shown to exist between the endogenous nitrogen metabolism and the basal energy metabolism of adult rats by Terroine and Sorg-Matter ('27) and by Smuts ('35) was established in a purely empirical manner. It amounts to about 2.0 mg. of endogenous nitrogen per calorie of basal energy expenditure under our conditions of estimating the two components of the ratio. The absence of unequivocal experimental evidence to the contrary has permitted heretofore the assumption that this ratio was constant under usual conditions. In the experiments above reported, statistical evidence has been presented showing that the relationship is subject to variation under some conditions. The plane of nutrition, by stimulating one component (basal metabolic rate) and not the other (endogenous output of nitrogen), causes a depression of the ratio. Low environmental temperature accompanied by increased caloric intake is without effect upon the ratio, since both components are almost equally stimulated.

CONCLUSIONS

The normal ratio for adult rats, observed in this laboratory, of approximately 2.0 mg. of endogenous urinary nitrogen per calorie of basal heat is disturbed under the conditions studied if they operate in such a way as to affect the two members of the ratio differently.

A prior low environmental temperature elevates the endogenous level of nitrogen excretion whether or not the plane of nutrition is simultaneously raised. On the other hand, such a lowering of temperature elevates the basal metabolic rate only when accompanied by an elevation in the

plane of nutrition (caloric intake) and then only to an extent no greater than could be produced by the raised plane of nutrition itself. Hence, the ratio of endogenous nitrogen to basal heat may be unaffected in the albino rat by a lowering of the environmental temperature if the plane of nutrition (caloric intake) is increased in proportion to the increase in energy requirements. It will always be depressed in the same animal if the plane of nutrition is raised with no concomitant lowering of the environmental temperature.

A different picture may be expected in animals whose basal metabolic rate is not so sensitive to changes in the plane of nutrition as is that of the albino rat.

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THE PATHOLOGY OF RIBOFLAVIN DEFICIENCY IN THE RAT [1]

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SIXTEEN FIGURES

(Received for publication June 5, 1941)

INTRODUCTION

Phillips and Engel ('38) have shown in the chick that a ration low in riboflavin results in a specific neuropathology of the main peripheral nerve trunks characterized by degenerative changes in the myelin of the nerve fiber, accompanied by Schwann cell proliferation, axis cylinder swelling and fragmentation. In rats on the same ration, Engel and Phillips ('39) were unable to demonstrate any similar changes. Wolbach ('37) had previously stated that there was no such degeneration in the rat on a vitamin B_2 low ration. In the dog he was able to demonstrate degeneration of the myelin sheaths in the peripheral nerves and in the posterior tracts of the spinal cord.

The occurrence of cataract in albino rats on a ration low in riboflavin has been described by various workers. Day, Darby and Langston ('37) and Day, Darby and Cosgrove ('38) showed the identity of riboflavin with the cataract preventive factor and the arrest of early nutritional cataract by the use of riboflavin.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

These studies were made possible by grants from the Wisconsin Alumni Research Foundation and the Works Progress Administration.

Recent investigations by Wagner, Axelrod, Lipton and Elvehjem ('40) and Mannering, Lipton and Elvehjem ('41) on the riboflavin requirement of the rat on synthetic rations have indicated that the riboflavin requirement of the rat is considerably greater on a ration high in fat than on a ration high in carbohydrate. On high fat rations, they were able to produce a more severe riboflavin deficiency characterized by spastic paralysis. A thorough investigation of the pathology of riboflavin deficiency in the rat on both carbohydrate and high fat rations which were strictly riboflavin deficient was undertaken to determine if there was any neuropathology or general pathology in the albino rat.

EXPERIMENTAL PROCEDURE

Two rations were used which were deficient in riboflavin. The first was a modification of ration K_{21} described by Wagner, Axelrod, Lipton and Elvehjem ('40) and consisted of the following:

	%
Dextrin	72
Alcohol ext. casein	18
Lard	3
Corn oil	3
Salts IV	4
	100

	PER 100 GM. RATION
Thiamine	300 μg.
Pyridoxine	300 μg.
Nicotinic acid	5 mg.
Pantothenic acid	2 mg.
Choline	200 mg.

The second ration was identical with the first with the exception that 49.5 parts of the dextrin were replaced isocalorically by lard. The fat content of this ration was 39%. Twice weekly, these rations were further supplemented by feeding 3 drops of haliver oil which contained 1 mg. of added synthetic α-tocopherol per 3 drop dose. Small quantities of ration were mixed as needed. The rations were stored in the refrigerator and fed daily to prevent rancidity.

Male rats ranging in weight from 60–80 gm. were distributed among four lots as follows:

LOT NO.	NO. OF RATS	PERIOD FED (IN WEEKS)	RATION
I (a)	5	8	Basal carbohydrate ration only
(b)	5	12	Basal carbohydrate ration only
II (a)	5	8	Basal carbohydrate ration ad libitum, plus 100 μg. riboflavin per day
(b)	5	12	Basal carbohydrate ration, intake restricted to that of individual rats in lot I (b), plus 100 μg. riboflavin per day
III (a)	5	4	Basal high fat ration only
(b)	13	8	Basal high fat ration only
IV (a)	5	4	Basal high fat ration ad libitum, plus 100 μg. riboflavin per day
(b)	13	8	Basal high fat ration intake restricted to that of individual rats in lot III (b), plus 100 μg. riboflavin per day

Group (a) in each lot was sacrificed early to study the progress of the pathology. The rats in group (b) of lots I and III were on experiment until severe deficiency symptoms appeared. When a riboflavin deficient rat became feeble, it was killed and autopsied. At the same time its mate in the control group was killed and a similar autopsy performed. Routine histological examinations were made of the adrenal, pancreas, testis, kidney, liver, spleen, pituitary, thyroid, thymus, stomach and small intestine, after they had been fixed in Bouin's fluid and stained in hematoxylin and eosin. Studies of the nervous system were made on the brain, the cervical and brachial segments of the spinal cord, the sciatic and inferior branch of the brachial plexus. The myelin sheaths of the sciatic and inferior branch of the brachial plexus were studied by means of plane polarized light between crossed nicol prisms (Setterfield and Sutton, '35) and by the Marchi osmic acid technique (Swank and Davenport, '34) after fixation in 10% formalin buffered at pH 7.0. The myelination of the cervical region of the cord was also studied by the Marchi technique. The axis cylinders of the brachial segment of the cord and the peripheral nerves were observed after silver impregnation by the Bodian method ('36). Nissl

preparations of the spinal cord and brain were made with gallocyanin according to Einarson's method ('32). All the tissues studied by either Bodian's silver or Einarson's gallocyanin methods had been previously fixed in a fluid consisting of 100 parts of 95% alcohol, 5 parts of glacial acetic acid and 2 parts of paraldehyde.

RESULTS

The rates of growth of the rats used in this experiment are shown in figure 1. It can be seen that when the rats were allowed access to the basal rations supplemented daily with riboflavin, good growth resulted. When the food intake of

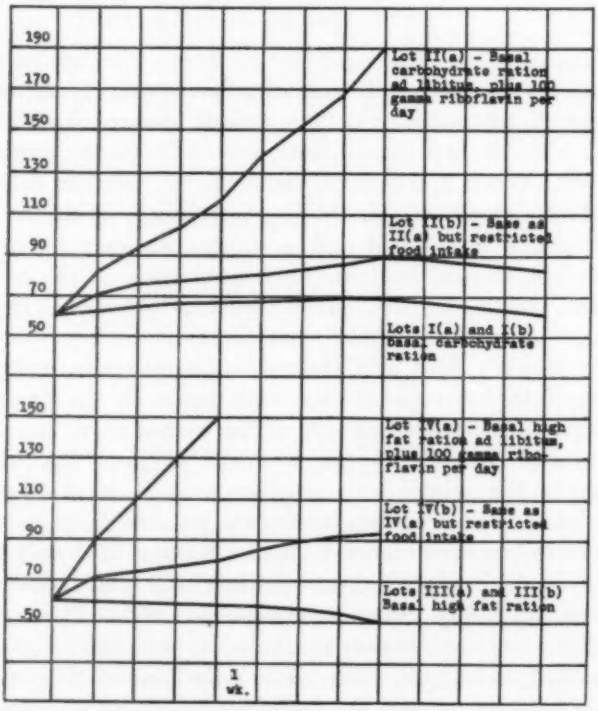


Fig. 1 Growth curves for rats raised on the carbohydrate and high fat rations.

the positive controls was restricted to the level of the riboflavin deficient rats, growth was considerably retarded.

The hair coat of the rats on the basal carbohydrate ration became rough and unkempt with a slight loss of hair. There was some dermatitis about the nose. In contrast, the rats on the high fat ration lost large amounts of hair so that sizable areas, particularly between the legs, under the belly and around the face, became almost completely denuded. The remaining hair was heavily matted together with lumps of the greasy ration. Dermatitis was much more severe in these animals. Considerable areas around the nose became necrotic and raw. In addition these rats developed a parchment-like skin and frequently, where it was wrinkled or folded, heavily encrusted ulcers. A slight hemorrhagic inflammation of the nasal mucous membranes of the deficient rats was common and resulted in a crusting of blood around the nostrils. The eyelids became sticky and there was noted a marked decrease in the amount of lacrimation which left the cornea quite dry at all times. Frequently both eyelids would stick together and the eyes would remain closed for several days. The eyes appeared to be swollen and slightly red. On the carbohydrate ration two cases of cataract developed. However six of the rats on the high fat ration developed a corneal opacity in one or both eyes.

With one exception locomotion was normal in all the rats on the basal carbohydrate ration. In the exceptional animal, a spastic gait developed which was also seen in eleven of the animals on the high fat ration. The remainder of the animals became weak and feeble without any gross evidence of a neurological disturbance (fig. 2). In those rats which developed a spastic gait, the hind legs became very stiff and were rigidly extended. Locomotion was very difficult due to the inability of the animal to flex the hind legs. Walking was accompanied by a side-to-side swaying motion produced when the body weight was shifted entirely to one leg while the opposite one was swung forward. Wherever the rat survived this spastic gait period for 2 or 3 days, the front legs ex-

Fig. 2 The rat on the right was fed the low riboflavin carbohydrate ration for 12 weeks. The rat on the left received the same ration with intake restricted to that of the former plus 100 μg. riboflavin per day.
Fig. 3 The rat on the right was fed on the high fat ration for 8 weeks. The rat on the left received the same ration with intake restricted to that of the former plus 100 μg. riboflavin per day.

hibited the same stiffness. The back of these rats was highly arched (fig. 3). The deficient animals were extremely restless. Even when spastic paralysis was severe, there was no attempt to rest. There was a slight increase in the food consumption during the latter stages of this type of paralysis.

The time of the onset of the severe symptoms varied by a period of several weeks on the two types of rations. Invariably the rats on the high fat ration began to show severe deficiency symptoms at the end of about 8 weeks, while those on the carbohydrate ration did not show severe symptoms before the end of 12 weeks.

The terminal stage in all these cases was a deep coma and death. In coma those which did not exhibit spastic paralysis were limp as though anesthetized, and the limbs of those with spastic paralysis were stiffly outstretched.

The rats on the basal rations when supplemented daily with 100 μg. riboflavin were normal in all respects.

The distribution of histological changes in the tissues of these rats is shown by table 1.

TABLE 1

Summary showing the frequency of histologic changes in various organs.

		NO. RATS	WEEKS ON EXPERIMENT	SCIATIC NERVE	INFERIOR BRANCH OF BRACHIAL PLEXUS	SPINAL CORD	TESTIS	THYMUS	THYROID	ADRENAL	CATARACTS
Lot I											
Basal carbo-	(a)	5	8	0	0	0	5	3	0	0	0
hydrate ration only	b)	5	12	1	0	0	5	5	4	3	2
Lot II											
Basal carbo-	(a)	5	8	0	0	0	0	0	0	0	0
hydrate ration plus crystalline riboflavin	(b)	5	12	0	0	0	0	0	0	0	0
Lot III											
Basal high fat	(a)	5	4	0	0	0	5	4	0	0	0
ration only	(b)	13	8	11	6	6	13	13	10	8	6
Lot IV											
Basal high fat	(a)	5	4	0	0	0	0	0	0	0	0
ration plus crystalline riboflavin	(b)	13	8	0	0	0	0	0	0	0	0

In those animals removed from experiment before paralysis had developed no neuropathology could be demonstrated by the techniques used. However, in those animals where paralysis was apparent a degeneration of the myelin of a few of the fibers of the sciatic could be demonstrated (figs. 5 and 7). In the early stages the inferior branch of the brachial plexus and the spinal cord were normal. When spastic paralysis became more severe, degeneration of the sciatic became very widespread with very few of the fibers remaining normal (fig. 6). At this stage a positive Marchi reaction was obtained in the cervical region of the spinal cord. This seemed to be localized mainly in the dorsal regions of the cord (figs. 8 and 9). Degeneration was also seen in the inferior branch of the brachial plexus. Where degeneration of the myelin sheaths had taken place in the sciatic and inferior branch of the brachial plexus fragmentation and bulbous swelling of the fibers could be shown with silver impregnation. In the regions of the cord where it was severely affected by these processes gliosis occurred. In the mild cases, there was only a slight positive Marchi reaction in the spinal cord unaccompanied by any repair process.

The testis in all the riboflavin deficient rats was atrophied, rather firm but retracted into the body cavity. This atrophy developed in the rats fed the basal rations for periods of 4 to 6 weeks. The epididymus was proportionately smaller and the prostate and seminal vescicles were grossly atrophied.

Fig. 4 Sciatic nerve. High fat ration plus riboflavin. Normal myelination. Marchi. × 440.

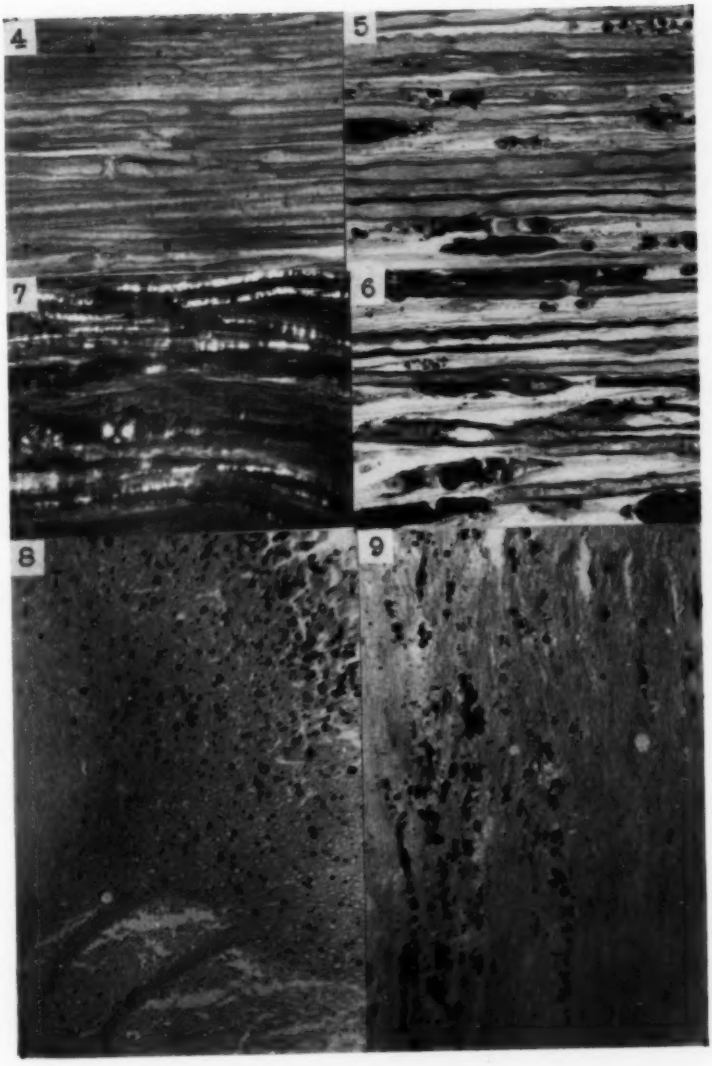
Fig. 5 Sciatic nerve. High fat ration only. Mild myelin degeneration in a few of the fibers. Marchi. × 440.

Fig. 6 Sciatic nerve. High fat ration only. Widespread myelin degeneration. Marchi. × 440.

Fig. 7 Sciatic nerve. High fat ration only. Degeneration of myelin as shown by disturbed distribution of birefringent materials in the myelin sheath. Crossed nicols and polarizing microscope. × 600.

Fig. 8 Transverse section of spinal cord. High fat ration only. Myelin degeneration in the dorsal paths of the cord. Marchi. × 200.

Fig. 9 Longitudinal section of spinal cord. High fat ration only. Myelin degeneration in the dorsal paths of the cord. Marchi. × 200.



Figures 4 to 9.

On histological examination of the testis, the seminiferous tubules were either undeveloped or atrophied. The lumina were much larger in relation to the diameter of the tubules than they were in the normal testis. In the early stages there was no evidence of spermatids, and there was a decrease in the number of meiotic divisions (fig. 11). Later the spermatogonial cells were clumped in the lumen of the tubule leaving the Sertoli cells lying bare against the basement membrane (fig. 12). The lumina of the tubules were commonly filled with detached spermatogonia in all stages of arrested development and degeneration. The epithelial cells of the tubules of the epididymus and their stereocilia appeared to be normal. The lumina of these tubules were filled with spermatogonia but no spermatozoa were seen.

The thymus in the riboflavin deficient rat had undergone an early involution which apparently had followed the normal course of aging. There was a decrease in the number of lymphocytes in the cortical region and then a replacement of these cells with adipose tissue (fig. 14). In the most chronic deficiency produced on the high fat ration the thymus had undergone complete involution and consisted of only a few lymphocytic cells surrounding the scattered Hassall's bodies.

In a few of the more severe cases the thyroid gland in the riboflavin deficient rat contained much less colloid than that of the control rats (fig. 16). The follicular epithelial cells

Fig. 10 Testis. High fat ration plus riboflavin. Normal tubules with meiotic divisions and maturing spermatids. H and E. × 440.

Fig. 11 Testis. High fat ration only. Early degenerative changes with decrease in the number of meiotic divisions and complete lack of spermatids H and E. ×440.

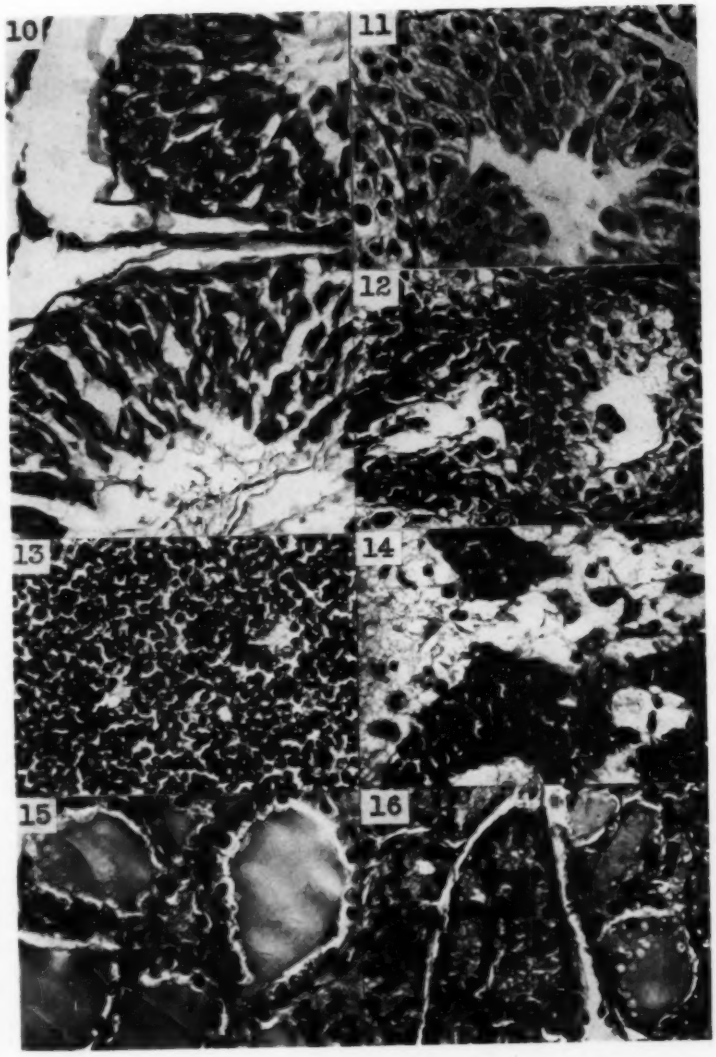
Fig. 12 Testis. High fat ration only. Late degenerative changes with complete disappearance of meiotic divisions, shrinkage of tubules. H and E. × 440.

Fig. 13 Thymus. Carbohydrate ration plus riboflavin. Normal number and distribution of lymphocytes. H and E. × 440.

Fig. 14 Thymus. Carbohydrate ration only. Decrease in number of lymphocytes and infiltration of adipose tissue. H and E. × 440.

Fig. 15 Thyroid. High fat ration plus riboflavin. Normal low cuboidal epithelial cells in acini with large colloid filled lumina. H and E. × 440.

Fig. 16 Thyroid. High fat ration only. High cuboidal epithelial cells in acini with reduced lumina and decreased amount of colloid. H and E. × 440.



Figures 10 to 16.

had undergone hypertrophy. The normal low cuboidal actively secreting epithelial cells were replaced by high cuboidal epithelial cells.

The adrenal in severe cases was also somewhat affected. The cells of the actively secreting area (zona fasciculata) of the cortex were scattered and diffuse in nature so that the cells did not lie in organized cords but resembled the distribution of cells in the post-secretory area (zona reticulata).

In all cases, the pancreas, kidney, liver, spleen, pituitary, stomach and small intestine appeared normal.

DISCUSSION

The deficiency of riboflavin in the ration of the rat produced a variety of pathological changes. The time of appearance and the severity of these changes varied depending on the type of ration used. When a carbohydrate ration deficient in riboflavin was fed, the symptoms appeared later and were much less severe than when this diet was replaced by an isocalorically equivalent high fat — low riboflavin ration. This fact has already been reported by Mannering et al. ('41).

These results demonstrate clearly that a riboflavin deficiency produced on a strictly deficient diet causes neuropathology in the rat similar to that in the chick (Phillips and Engel, '38). This probably explains the results previously obtained by Engel and Phillips ('39) who reported no histopathology in the nervous system of the rat fed a low riboflavin diet. In addition to the changes in the nervous system it is quite apparent that other structures are damaged early in the development of a riboflavin deficiency in the rat. The thymus, thyroid, and testis show histologic changes earlier than neural lesions. It would appear that riboflavin is utilized by the body at the expense of these tissues.

It must be kept in mind that these histopathologic changes produced on riboflavin deficient rations may be secondary in nature but at least they are always associated with the histopathology of this deficiency.

These researches tend to emphasize again the interrelationship of riboflavin and lipid metabolism, a fact which is supported by the work of Phillips and Engel ('38) and Mannering, Lipton and Elvehjem ('41). The demonstration that such cellular structures as the testis and thymus are affected is suggestive of a more intimate relationship with cellular metabolism. This might well be concerned with phosphorylation as suggested by Mannering et al. ('41).

SUMMARY

It has been demonstrated that a severe riboflavin deficiency in the rat results in a partial paralysis of the legs. This type of paralysis is produced more easily on a high fat ration and it is prevented by the daily feeding of crystalline riboflavin. This paralysis in its severe form is characterized by degeneration of the myelin sheaths of the nerves accompanied by axis cylinder swelling and fragmentation. Myelin degeneration and gliosis in the spinal cord have been observed.

Histologic examination of the testis and thymus of riboflavin deficient rats shows that there is an early and marked atrophy of the testis and an abnormally early involution of the thymus. Structural changes are also observed in the thyroid and adrenal of the most severe cases. These changes have been described.

It is concluded from these studies that a riboflavin deficiency in the rat causes histopathologic changes in certain endocrine glands, the testis and also in the central nervous system.

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VITAMIN B COMPLEX AND FAT METABOLISM

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McHenry and Gavin ('40) showed that the administration of a beef liver concentrate to rats on a fat-free diet, containing adequate amounts of thiamine, riboflavin and pyridoxine, led to a marked increase in liver fat and cholesterol which was prevented by lipocaiac, but not choline. Recently these authors have reported that the addition of pantothenic acid to the vitamin supplement was not sufficient to produce the choline resistant fatty liver but, if biotin was given in addition, choline-resistant fatty livers were produced ('41).

Studies on the effect of the B complex vitamins on fat metabolism have been in progress in this laboratory for over a year and the results obtained with pantothenic acid are in complete agreement with those of McHenry and Gavin. However, contrary to the observations of Gavin and McHenry ('40) we have found that the administration of nicotinic acid renders the liver lipids somewhat more resistant to the lipotropic action of choline. Also, under the conditions studied, the administration of this vitamin definitely increases the cholesterol content of the liver. The experimental conditions differed, however, from the work of the above authors with nicotinic acid in that our diet supplied adequate amounts of pantothenic acid while theirs contained none. This difference in supplements may account for the difference in results.[1]

Young rats of both sexes, weighing from 70 to 90 gm., were used. All analyses were made on individual livers, but as no

[1] The vitamin preparations used were kindly supplied by Merck and Company.

difference was found between the sexes, the results are pooled irrespective of sex in the accompanying tables. All of the animals were put on one of the basal diets given in table 1 for a period of 2 or 3 weeks before addition of the supplements.

TABLE 1

Basal diets

	DIET 1	DIET 2
	gm.	*gm.*
Casein, vitamin free, Labco	10	10
Sucrose	83	83
Salt mixture	4	4
Agar	2	2
Cod liver oil	1	—
Porcomorph oil	—	0.03
Supplemented by		
Thiamine chloride	0.2 mg.	0.2 mg.

The methods employed for the liver analyses were the same as described previously by Outhouse and Forbes ('40). For the determination of body lipids, the intestinal tract, liver, skin, feet and tail were removed and the carcass ground in a meat grinder. After grinding, the carcasses were weighed, placed in an oven at approximately 100°C. and dried for several days. The material was then reweighed, ground finely in a mortar and samples taken for analysis. The weighed sample, usually about 1 gm., was again ground with approximately 5 gm. of a commercial zeolite,[2] transferred to a 125 cc. glass-stoppered bottle and 100 cc. of chloroform added. It was then set aside with occasional shaking for at least 24 hours, after which aliquots were taken for analysis. All livers were analyzed separately, but the bodies were usually run in groups of two to four. It would be preferable to determine the lipid content of the whole body but it was found impossible to obtain satisfactory samples for analysis if the skin was not removed before the bodies were ground. However, since the same procedure was used in all of the experiments, the results

[2] Doucil. Material used in fat analyses was obtained from W. A. Taylor and Company, Baltimore, Maryland.

are comparable. In two sets of experiments the lipid content of the dried skin was determined. It was found that in each of the experiments the neutral fat plus cholesterol content varied more or less directly with that of the rest of the body. Consequently, it seems likely that the same general relationship in lipid values would have been obtained if the whole body had been used for analysis.

In experiments 6 and 7, in order to eliminate fat completely from the diet, the vitamin A and D preparation was discontinued during the feeding of supplements. In experiments 11, 12 and 14, 50% porcomorph oil[3] was used to the extent of 0.03% of the diet throughout the experimental period. Since *l*-cystine is known to increase the deposition of fat in the livers of rats on a high fat-low choline diet, it was decided to add this amino acid to the fat-free diet, in certain of the experiments, in the hope that under these conditions it might favor the deposition of fat in the livers of the experimental animals. Consequently in experiments 5, 6 and 7 the diet included 0.5% cystine during the supplement feeding period. In experiments 11 and 12 the diet included 0.1% cystine throughout the experimental period. In all experiments the vitamin supplements, when fed, were given at the following levels, pyridoxine 500 μg., riboflavin 500 μg., calcium pantothenate 1600 μg. and nicotinic acid 1600 μg. per 100 gm. of food. In experiments 4, 5, 6 and 7 the choline-fed rats received 10 mg. of choline chloride per rat per day. In experiments 12 and 14 choline chloride was incorporated in the diet to the extent of 10 mg. per 10 gm. of food, and the average food consumption exceeded 10 gm. per day.

DISCUSSION

The experimental results recorded in table 2 show that the administration of either pyridoxine, riboflavin or calcium pantothenate, in addition to thiamine, exerts no demonstrable effect upon the concentration of neutral fat or cholesterol in the liver, over that produced by thiamine alone. That pyridoxine has no effect, was demonstrated previously by Gavin

[3] Mead Johnson.

TABLE 2

Effect of various supplements on the cholesterol and neutral fat content of the body and liver.

EXPERIMENT NO.	NUMBER OF RATS	AVERAGE DAILY GAIN IN WEIGHT DURING SUPPLEMENT FEEDING PERIOD	LIVER				MINCED BODY TISSUE		SUPPLEMENTS
			Neutral fat	Cholesterol	Neutral fat per 100 gm. of body weight	Cholesterol per 100 gm. of body weight	Neutral fat	Cholesterol	
		gm.	*%*	*%*	*gm.*	*gm.*	*%*	*%*	
11	5	—0.7	10.4	0.42	0.538	0.022	3.12	0.18	31 days on diet 2.
	6	+0.1	10.9	0.45	0.556	0.023	3.10	0.20	21 days on diet 2. 10 days on Ca pantothenate in addition.
	4	—0.4	13.0	0.46	0.619	0.023	3.31	0.19	21 days on diet 2. 10 days on B_2 in addition.
	3	—0.2	11.6	0.55	0.620	0.031	3.93	0.17	21 days on diet 2. 10 days on B_6 in addition.
4	7	+1.1	16.4	0.34	0.995	0.021	5.82	0.18	21 days on diet 1. 10 days on B_2, B_6 and Ca pantothenate in addition.
	5	+1.2	3.7	0.26	0.160	0.012	7.62	0.18	Same plus choline during supplement feeding period.
	3	—0.2	5.4	0.22	0.264	0.010	3.89	0.21	21 days on diet 1. 10 days on B_2 and B_6 in addition.
	5	+0.5	1.0	0.17	0.043	0.007	5.30	0.18	Same as above plus choline during suppl. feeding period.
5	4	+0.9	25.1	0.65	1.666	0.045	4.22	0.18	21 days on diet 1. 10 days on B_2, B_6 and Ca pantothenate in addition.
	5	+1.8	5.0	0.41	0.204	0.018	5.31	0.19	Same as above plus choline during suppl. feeding period.
	5	+0.8	18.6	0.33	1.045	0.019	4.22	0.18	21 days on diet 1. 10 days on B_2 and B_6 in addition.
	5	+1.3	6.8	0.30	0.314	0.014	4.41	0.19	Same as above plus choline during suppl. feeding period.
6	5	+1.3	25.9	0.47	1.922	0.035	3.51	0.19	14 days on diet 2. 9 days on B_2, B_6 and Ca pantothenate in addition.
	5	+1.5	2.7	0.24	0.151	0.014	3.80	0.20	Same as above plus choline during suppl. feeding period.
	5	+2.0	16.5	0.33	0.959	0.019	3.03	0.17	14 days on diet 2. 9 days on B_2 and B_6 in addition.
	5	+1.6	2.6	0.19	0.118	0.009	3.40	0.20	Same as above plus choline during suppl. feeding period.
7	5	+1.0	18.7	0.66	1.001	0.037	5.21	0.19	14 days on diet 2. 11 days on B_2, B_6 and Ca pantothenate in addition.
	5	+0.4	1.5	0.25	0.062	0.010	3.05	0.15	Same as above plus choline during suppl. feeding period.
	5	—0.7	4.9	0.38	0.175	0.015	1.61	0.19	25 days on diet 2. No A or D during last 11 days.
	5	—0.8	1.0	0.31	0.035	0.011	1.50	0.20	14 days on diet 2. 11 days on Ca pantothenate without B_1.
12	9	+2.9	22.2	1.47	1.511	0.098	6.83	0.17	21 days on diet 2. 10 days on B_2, B_6, Ca pantothenate and nicotinic acid in addition.
	9	+3.2	6.4	0.77	0.305	0.036	7.23	0.17	Same as above plus choline during suppl. feeding period.
14	5	+1.3	11.8	0.75	0.659	0.042	5.80	0.22	21 days on diet 2. 10 days on B_2, B_6, and Ca pantothenate in addition.
	7	+1.4 [1]	15.7	1.53	0.875	0.085	6.10	0.25	21 days on diet 2. 10 days on B_2, B_6, Ca pantothenate and nicotinic acid in addition.
	5	+2.0	5.6	0.62	0.275	0.030	7.80	0.23	Same as above plus choline during suppl. feeding period.

[1] If the value for one animal which grew very little is excluded, this value becomes 1.7.

and McHenry ('40). The present investigation shows that the administration of both riboflavin and pyridoxine increased as a rule the neutral fat content of the liver but exerted no definite effect upon the concentration of liver cholesterol. When, in addition, calcium pantothenate was given, the neutral fat content was further increased, and at the same time the concentration of liver cholesterol was also raised. When nicotinic acid was given in addition to these, the effect on liver cholesterol was very striking, the total amount in the liver being in some cases increased to about ten times the normal value. On the other hand, the concentration of neutral fat was not materially increased over that obtained when thiamine, riboflavin, pyridoxine and calcium pantothenate only were given. The body neutral fat content, however, was definitely elevated over that obtained in the absence of nicotinic acid. Choline administration exerted a definite lipotropic action under all conditions studied. On the whole, however, this effect was definitely less marked in those animals receiving nicotinic acid. The feeding of choline also reduced the deposition of cholesterol in the livers. In the case of the nicotinic acid-fed animals, however, the liver cholesterol values remained quite high, on the average, in spite of choline administration. These results suggest that nicotinic acid might be a factor in the production of choline-resistant fatty livers. However, since Gavin and McHenry ('40) have shown previously that choline will prevent fatty livers when the animals are fed a diet containing nicotinic acid, thiamine, riboflavin and pyridoxine but no pantothenic acid, it would appear that no particular factor of the vitamin B complex is directly responsible. It is probable that all of them may influence the final result. It may be significant that in all experiments in which choline-resistant fatty livers have been reported the rate of growth of the animals during the supplement feeding period was quite rapid (McHenry and Gavin, '40, '41; Engel and Phillips, '39). Since a rapid rate of growth is usually associated with the consumption of a large amount of food, it is possible that a fairly large carbohydrate intake is one of the essential factors in the production of choline-resistant fatty livers.

[1] If the value for one animal which grew very little is excluded, this value becomes 1.7.

SUMMARY

The administration of thiamine, riboflavin, pyridoxine, nicotinic acid and calcium pantothenate to rats on a fat-free, 10% casein, high carbohydrate diet led to the production of fatty livers containing quite a high concentration of cholesterol. When nicotinic acid was absent from this diet the animals developed fatty livers whose cholesterol content, though elevated, was low in comparison with that obtained when nicotinic acid was also fed. Addition of choline to the diet exerted a definite lipotropic action under all conditions studied. However, its effect as a whole was less pronounced in those animals receiving nicotinic acid than in the others.

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DIETARY PRODUCTION OF CATARACTS IN LARVAL AMBLYSTOMA TIGRINUM

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ONE PLATE (NINE FIGURES)

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The small vertebrates of the genus Amblystoma have been used extensively in analyses of various general principles of early vertebrate morphology and physiology. The author has found a striking resemblance between the dietary responses of the rapidly-growing larvae of A. tigrinum and those of higher vertebrates. The larvae are highly sensitive to dietary factors influencing growth and development. In addition, diseases common to man are easily produced in the small urodeles by the same dietary irregularities causing those diseases in the human form. Faulty calcification, tetany, anemia, and cataracts have resulted from different dietary inadequacies. Two types of lens changes have appeared (Patch, '34, '41), and these have consistently followed use of diets differing in the protein ingredient, the chief food requirement of these carnivores. Because of this specific relation and also the similarity between the lens changes in the salamander larvae and in man, these animals are excellent subjects for studying etiology of the disease.

A. NORMAL LENSES IN ANIMALS FED BEEF MUSCLE DIET

A diet with powdered beef "round" as chief component, serving as a calcium-low basis for the study of needs of

salamander larvae for the calcifying minerals,[1] produced normal lenses and hence served as a control diet in the study of cataract formation. The basal ration contained powdered beef (63%), fat[2] (5%), cod liver oil (2%), bakers' yeast (1%), and cornstarch (29%) cooked into a paste for binding the ration (Hubbell and Mendel, '27). In the first experiment recorded here ('33), the beef ration was fed to forty-six larvae of the species A. tigrinum raised from eggs of an Illinois strain. The animals were in five groups given different combinations of calcium, phosphorus, and other mineral nutrients. In each of these groups the eyes were normal, the lenses appearing black by reflected light (fig. 1), colorless and transparent by transmitted light. No lens disturbance could be detected either by binocular examination or by histological methods. This diet produced good growth and an adequate blood supply and, when properly supplemented, led to development through normal metamorphosis to the adult form.

B. CATARACTS CAUSED BY A DIET OF PURIFIED CASEIN AND POWDERED MILK

A diet of casein and powdered milk (table 1) was used to determine whether the anemia previously observed in salamander larvae fed this diet (Patch, in press) was due to deficiency of iron and copper as had been found true of the widely-studied milk-anemia of rats. In order to have a minimum of minerals in the basal ration the chief ingredient, casein,[3] was carefully prepared. This protein, separated from

[1] The content of the foodstuffs, minerals and vitamins used in the rations for the salamander larvae was determined by their response in preliminary experiments with different proportions of those materials (Patch, in press), and by reference to analyses of the natural early diet of the larvae which consists largely of Entomostraca. The plant-characteristic vitamin C was not given to the flesh-eating salamanders. "Maximum feedings" were given, as used by Springer ('09) in growth studies of the newt, and in correspondence with the "ad libitum" feeding of experimental rats. All groups of animals in a series of comparisons were fed by the same time schedule.

[2] Wesson oil.

[3] W. R. Todd supplied the purified casein used in 1933 and 1934. Frank Stirn and Edwin Hove supplied that used in 1935 and 1936.

skim milk, was given seven or more alternations of solution by dilute alkali and precipitation by acid (Todd, Elvehjem and Hart, '34; Hubbell and Mendel, '27). The diet was given to forty-two A. tigrinum larvae of the same lot as those fed the muscle diet. The animals were divided into six groups, each with a different combination of iron and copper levels.

Before the end of 2 weeks, growth of these larvae had lagged behind that of those fed the muscle ration, and during the seventh week those fed the casein showed a poor appetite and a weakness of movement. Eleven had died before it was realized that the low food intake was partly due to poor vision caused by clouding of the lens. During the following 5 weeks,

TABLE 1

Composition of the casein ration.

INGREDIENTS	PROTEIN	CARBOHYDRATE	FAT	MINERALS	VITAMINS	TOTAL
	%	%	%	%		%
Casein	45	..	..	..	..	45
Whole milk powder	10	14	11	2	A, B, G	37
Cornstarch	..	15	..	..	..	15
Cod liver oil	..	..	2	..	A, D	2
Yeast powder	1	..	..	..	B, G	1
Total	56	29	13	2		100

eleven cases of cataract (36%) developed in the thirty-one surviving animals. Increase of both vitamin supplements failed to improve the health of the larvae. Seven were killed and preserved for histological study, four with and three without visible cataracts. Two with the disease were kept alive by transfer to an adequate diet, so that the late stages of the eye changes might be observed. The rest (seventeen) died before showing cataracts, although undoubtedly at least some of them had the disease in an early stage.

Cataracts occurred with a similar frequency in larvae fed the casein diet made with a low fat content by use of skim milk powder. The only reaction to later increase in supply of fat and vitamins was a slight and transient acceleration of growth. Addition of autoclaved yeast to the diet of nine

larvae, to give them six times the original yeast supplement, did not relieve their general debility as noted by Day, Langston and O'Brien ('31) in rats when vitamin G was increased in the cataract producing diet of Sherman and Spohn. Four cases of cataract appeared (66%) among six salamander larvae living when the first case was seen, the last case appearing 7 weeks after the yeast was increased. The other two members of the group died before showing the disease. Four cases of cataract (40%) also appeared among ten larvae given the low fat ration with its vitamin A in the form of carotene doubled after 7 weeks.

The cataracts. An early stage of the cataracts was seen rarely (fig. 2) as striae between the equator of the lens and its anterior pole. Usually, the disease did not become evident until the swollen and whitened lens protruded into the anterior chamber of the eye (fig. 3). Dissection showed that the disease was confined to the cortical layers of the lens, and this was confirmed by histological examination.[4] The rate of progress of the disease varied in different animals and bilaterally in the same animal. The gradual decrease of the whiteness of the pupils of the eyes as seen by unaided vision was found to be due to disappearance of the shrinking lens (fig. 4). There was seen one case of bilateral subluxation (figs. 5, 6) which allowed direct observation of the lenses throughout the late stages of the disease.

Absence of cataracts in larvae fed additional cystine. It was suspected that the cause of the general poor health and of the cataracts in the salamander larvae lay in the purified casein, chief ingredient of the faulty ration. As the casein was not toxic to rats, its fault was assumed to lie either in its composition or in its utilization by the larval salamanders. Nine larvae fed the low fat ration were also given a low intake of protein (2.4% of the purified casein; total protein 16%) with the carbohydrate increased by dextrin to 78%. No growth occurred in these animals and hence no faulty lens protein was formed; neither did any deaths occur. An

[4] The histological conditions will be discussed elsewhere.

edema appearing in the sixth week was relieved by increase of the dietary fat to the usual level, but no other response was evident. Two weeks later, the purified casein was increased to give three protein levels of 30, 55, and 80% each for three larvae. All of the animals began to grow, with the greatest acceleration in those given the middle level. The low content of cystine in casein has long been known to be of biological significance to rats. Accordingly, cystine was added to the diet in the amount which would be present if the dietary protein were beef muscle (Sherman, '37). When the supplement of cystine was started, 2 weeks after growth had been initiated by the protein increase, one case of cataracts was developing in the group. No other case appeared, although these larvae lived longer than those which were fed the higher level of purified casein from the beginning and which suffered early from its inadequacies.

Repetition with a different strain of A. tigrinum. Although the cataracts seen in the casein-fed salamander larvae were quite clearly a result of diet, there was a possible relation to strain of the animals. To eliminate this the above work was repeated with two sets of larvae of the same species raised from Wisconsin eggs ('34, '35). In both sets, the diet of purified casein and powdered milk produced cataracts like those previously seen, and no cataracts developed in larvae fed this same diet supplemented with cystine. Other diet groups free from cataracts included seventy-two larvae fed the synthetic diet of beef muscle with different combinations of vitamin D, calcium, and phosphorus (Patch, '35), six larvae given unrefined casein with the milk powder, fifty-four given the diet of purified casein with six different commercial liver extracts (Patch, '36), ten fed raw beef muscle, and fourteen fed raw beef liver.

C. CATARACTS PRODUCED BY DIETS OF THE SEPARATE MUSCLE PROTEINS

The results of the above experiments indicated that the cataracts produced in the salamander larvae by the diet of purified casein and powdered milk were due to qualitative

inadequacy of the dietary protein. This was apparently true also of the anemia. Neither of these diseases had appeared in the species when fed the beef muscle ration. However, beef muscle contains several proteins, some fat and minerals, as well as vitamins of the B complex group. In order to use isolated proteins, yet some which were more closely allied in composition to those of the natural diet of the carnivorous salamanders than was the casein of the early experiments, muscle was fractionated and separate diets were made of its most abundant proteins. Quickly-ground cheek muscle from newly-killed cows was extracted with an alkaline-buffered KCl solution at a temperature just above freezing, and the dissolved proteins were precipitated separately by graded dialysis against buffers of progressively lower pH (Weber and Meyer, '33). The precipitated myosin, and then the globulin x in turn, were washed with the precipitating buffer solution to remove traces of the proteins soluble at that pH. Each of these proteins, as well as the extraction residue (chiefly stroma protein), was first washed with distilled water to remove inorganic ions and then was dried and pulverized. The third protein precipitated, namely water-soluble myogen, is accompanied in red muscle by an approximately equal amount of hemoglobin of muscle and of blood not separable from the myogen. To prepare this muscle protein in purer form, the suggestion of Weber was followed and extraction made of the hemoglobin-low white muscle of chicken.[5] The precipitated myogen (Weber, '25) was dialyzed against water to remove inorganic ions, and then pulverized. The three powdered proteins from beef muscle and the four prepared from white muscle of chicken were used as the proteins of seven different rations (table 2), which were fed to Amblystoma

[5] The amounts of the muscle proteins obtained from this preparation may serve to illustrate the relative quantities of the four proteins regained and the food quantity required for a set of experimental diets for the salamander larvae. From the white muscle of two chickens, totalling 8 pounds in weight, there were obtained in pulverized form 8.67 gm. of myosin, 6.09 gm. of globulin x, 6.65 gm. of myogen, and 9.77 gm. of the extraction residue.

tigrinum larvae raised from eggs of Tennessee ('36, '37, '38)[6] and of Wisconsin ('39, '40).

Cataract production. Comparison was first made of the nutritive value of the extraction residue and the myosin prepared from beef muscle. The larvae fed the extraction residue were small, but well-proportioned and active; they were not anemic and half of the group metamorphosed to the adult state. In contrast to animals fed whole muscle, however,

TABLE 2

Composition of the diet for protein variation.

INGREDIENTS	PROTEIN	CARBOHYDRATE	FAT	MINERALS	VITAMINS	TOTAL
	%	%	%	%		%
Protein	60	..	..	..	..	60
Cornstarch	..	27	..	..	..	27
Oil (Wesson)	..	..	10	..	..	10
Minerals [1]	..	..	..	3	..	3
Vitamins [2]					ABDG	
Total	60	27	100	3		100

The non-varying ingredients were mixed in quantity sufficient for 100 gm. of ration, and the mixture was kept frozen in 10 gm. lots until time for use. Before adding to the other ingredients of this mixture, the cornstarch was cooked (Hubbell and Mendel, '27) with a minimum of distilled water to make a paste. In making a ration, the proper amount of the paste mixture was added to the protein and other variants. Distilled water was added to obtain the suitable consistency for feeding.

[1] Osborne and Mendel ('18).

[2] In early work, vitamins were supplied by cod liver oil (2%) and powdered bakers' yeast (1%). Later, Abdol was used, a capsule supplying 100 gm. of ration with 6200 U.S.P. XI units of vitamin A, 900 of the same units of vitamin D, 45 Sherman units of vitamin B, and 10 Sherman units of vitamin G(B_2).

cataracts developed in four of six living for 2 months or more on the muscle fraction. The other members of the group died without showing the eye disease. The cataracts produced were like those caused by the diet of purified casein and powdered milk, but here the disease was clearly revealed to be independent and primary. In contrast to the good health

[6] The Tennessee eggs were supplied by Dr. R. C. Hutchinson of The Morris Biological Farm of The Wistar Institute.

of the larvae fed the residue diet, those fed the myosin were severely anemic and began to die in the fifth week before any cataracts had appeared. Five remaining animals were fed extraction residue together with the myosin to approximate the relative proportions of these two proteins in whole muscle (Weber and Meyer, '33). Three developed to metamorphosis which was successful in two. Two cases of cataract appeared, one in a metamorphosing animal. The relation of the myosin to the cataracts was not known until larvae of another experimental lot were maintained longer on a diet with myosin as the only protein, and cataracts appeared in one of two surviving the longest. Cataracts were also produced in this second set of animals by a diet of beef globulin x, which was poor for growth and development, but did not cause a marked anemia as the myosin did. Production of cataracts by diets of all four proteins prepared from the white muscle of chicken completed the evidence that one or more substances needed for formation of normal lens protein had been removed from the whole muscle during the extraction process and had not been recovered with any of the separated proteins.

Control diets not associated with cataracts. Other larvae of the same origin as those fed the separate muscle proteins were fed artificial diets of powdered whole muscle of beef that had not been treated and diets of powdered muscle that had been denatured first by cooking or by spoiling after alternate freezing and thawing. No cataracts developed in these larvae, in others fed raw beef liver, or those given artificial diets containing liver, halibut muscle, red or white muscle of chicken.

Preventive experiments. The cataracts caused by the diets of the muscle proteins exhibited in their development the same changes as the cataracts due to the purified casein diet. Visibility of an early stage was rare, so that curative experiments were not possible. Prevention was not attained by dietary supplementation with riboflavin (1.25 μg. per gram of ration), the factor which Day, Darby and Cosgrove ('38)

found effective in preventing cataracts in rats fed the casein and cornstarch diet with low vitamin G content. For these reasons, and because both the casein purification and the muscle extraction had included treatment with dilute alkali, it was thought that there might be a unity of cause for the production of the eye disease by the two sets of diets. Possibly, cystine had been removed during the alkaline-buffered extraction of the muscle proteins as during the treatment of casein by dilute alkali (Jones and Gersdorff, '34). Accordingly, cystine supplementation was tried with the muscle-protein diets. Prevention was not attained when the amount of this amino acid (1.25% of the dietary protein) which had been effective with the diet of purified casein and powdered milk was added to the diet of the residue from the beef extraction. Three cases of cataracts developed among six survivors, while three cases also appeared in a group of five larvae living on the diet without cystine. No cataracts appeared in a second group of five larvae given this diet with twice the earlier supplement. Also, none appeared in a group fed beef globulin x with this amount of the supplement. However, when this supplementation was repeated with larvae of another experimental lot and with muscle proteins of another extraction, complete prevention was not obtained with either the diet of the extraction residue or that containing myosin. Two cases of lens disease developed among six animals surviving longest on the former diet with cystine and one case among three fed the supplemented myosin. Possibly these animals needed a greater supply of cystine. In a group of five larvae of the same lot fed the myosin diet without the cystine, two cases of cataract appeared, one showing unilateral subluxation. The number of cataracts in larvae fed the seven muscle-protein diets, with and without the experimental supplements, was doubtless greater than the total seen (twenty-eight in fifty-five larvae or 51%), because some of the animals that died probably had the disease in an early non-visible stage.

D. CATARACT PRODUCTION BY DIETS OF HIGH HEMOGLOBIN CONTENT

At the same time the diet experiments with the muscle proteins were started, groups of the flesh-eating salamander larvae were also placed on different diets of powdered beef-blood preparations whose nutritive values were not known because they had been found distasteful to rats (Mitchell and Hamilton, '29). The rations (table 2) were similar to those of the muscle proteins, with protein supplied by whole blood, defibrinated blood (beef and also turtle), hemoglobin (Bradley and Sansum, '14), or fibrin. The larvae fed these diets grew slowly and did not progress far toward metamorphosis, but they clung to life tenaciously.

Cataracts of type two. Diets of the first three blood preparations produced cortical cataracts, but these were of a type very different from those caused by the diets previously described. All of the larvae (100%) fed the diets of high hemoglobin content showed a zoning of the lenses, sometimes evident after a month on the rations. A line of demarcation (fig. 7) inside a clear zone at the lens surface appeared to grow darker and broader. Sometimes there were two or three of these lines parallel to the lens surface, the outer ones appearing fainter. After an extended stationary period, the banding disappeared in 37% of the larvae and was replaced by whitening of the lens cortex (fig. 8). With the whitening, occurring 2 to 6 months after the diet was started, there was a large amount of gelatinous fluid within the lens capsule. This was followed by another period of slow change, during which the cortex grayed gradually, but lost little in size; the lens position was not changed, and the subcapsular fluid was retained. The bilateral difference in rate of progress of the disease was less than that which occurred with cataracts of the first type. The histological changes of the two types of cataract were strikingly different.[1]

The lens zoning did not appear in the larvae fed blood fibrin, nor in those fed a diet of beef muscle one-fourth replaced by

[1] See footnote 4, page 368.

heme (Anson and Mirsky, '30) and thus containing more than three times the amount of that pigment in the ration of hemoglobin. The line did not appear in the small larvae fed a similar diet of heme and myosin; but cataracts typical of a myosin diet developed in the larva living longest. If the cataracts produced by the blood diets had been due to the heme pigment, the presence of more than the natural amount of heme should have brought cataracts of the second type, regardless of the attending protein. A faint line of demarcation appeared near the lens surface in the larvae maintained without growth by a diet of the inseparable mixture of the hemoglobins of muscle and blood with the water-soluble myogen extracted from beef muscle. The same faint zoning appeared in eyes of the larvae losing size on a diet of separated denatured globin and heme in the natural proportion, and in similar larvae fed a diet of the globin without heme. These larvae survived to the point of development of mature cataracts only if transferred to the more nutritious diet of dried blood.

Effect of diet change in suppressing cataract development. Because the second type of cataract always had a visible early stage, attempts to cure the disease or to halt its progress were feasible. When larvae with a definite lens zoning were placed on a diet adequate for lens health, such as raw muscle or raw liver, the line in the lens did not disappear, but became buried by a band of uninterrupted transparency (fig. 9) which halted progress of the diseased condition. The width of the newly-formed zone of normal tissue was related to the growth value of the new diet, and was greatest with liver feeding.

Preventive factors. Lens zoning and the subsequent development of cataracts in larvae fed the rations of high hemoglobin content were not prevented by a supplement of riboflavin (1 μg. per gram of ration) nor by methionine (2.5% of the total protein). High supplementation with cystine (7% of the total protein or 5% of the total solids of the ration) was ineffective. In contrast to casein, globin is poorly supplied

not only with cystine, but also with the other amino acids present in glutathione. This tripeptide, long recognized to be important in lens metabolism (Krause, '34), has been found to be most abundant where new lens fibers are forming. When a group of larvae was fed the diet of whole blood supplemented with glutamic acid (15% of the protein) and cystine (3.3%), a definite lens zoning had appeared in only one-fourth of the animals at the end of a month and this proportion was held through 6 months with no further development of the disease. In larvae given the blood diet with glutamic acid and cystine, plus glycine (3%) as well, the zoning was further retarded and vague. The addition of arginine (4%), which is present in globin in small amount (Bergman and Niemann, '37), with the other three amino acids did not enhance their effect. That the action of the three acids was partly due to cystine was proved by an earlier and more definite lens zoning in larvae receiving the blood diet supplemented with glutamic acid and glycine but no cystine. Failure to secure complete prevention of cataracts by a liberal supply of the three acids suggested an inadequacy of these acids in the uncombined form or an insufficiency of some other dietary component. Development of the second type of lens disorder, rather than that produced by the casein diet (containing abundant glutamic acid and a fair amount of glycine) with no cystine supplement, gave evidence that an undetermined irregularity of the diet had been directing the specific nature of the lens changes.

DISCUSSION

Need for a cystine supplement with the diet of purified casein and not with that of unrefined casein was explained by the data of Jones and Gersdorff ('34) who found that repeated treatment of casein by means of dilute alkali not only removes the cystine adhering to the casein in its separation from the lactalbumin of milk, but also causes a disintegration of the cystine inherent in the casein molecule. In the present experiments the cystine supplement acted specifically on the formation of lens protein and did not hasten growth or prevent

the anemia. Other evidences of a specific relation of this amino acid abundant in ectoderm to the health of the ectodermally-derived lens have been reported in work with other animal forms. When Day, Langston and Cosgrove ('34) replaced the casein of the Sherman and Spohn diet deficient in vitamin G by the higher cystine-containing egg albumin, no cataracts appeared in their experimental rats although a marked dermatitis developed. These workers also found that chickens fed a diet with egg albumin as the source of protein had severe dermal lesions and only slight eye changes, while chickens given the same diet with casein showed cataracts and only a mild dermatitis. Bourne and Pyke ('35) reported an absence of cataracts in rats fed the regular diet of Sherman and Spohn when it was supplemented by cystine (1%). Bourne and Young ('34) expressed the opinion that the cause of naphthalene cataracts in rabbits might be due to the fact that these animals detoxicate this aromatic hydrocarbon by a conjugation with cysteine, thus depriving the lens of that amino acid. Yudkin and Geer ('40) reported prevention of cataracts due to high galactose rations (Mitchell, '35), when a high supplement (4%) of cystine was used. In the ration of purified casein and powdered milk, which produced cataracts in the salamander larvae, the galactose content was low (7%). Moreover, the same galactose percentage was present in the diet when the preventative cystine was added and the same amount was present with the preventive diet of commercial casein. No galactose was included in the diets of the separate muscle proteins nor in the diets of high hemoglobin content; all of these diets produced cataracts.

The primary nature of the lens disease, shown by the specific action of cystine as a supplement to the diet of purified casein, was emphasized by the occurrence of cataracts in animals developing normally through metamorphosis on the diets containing the stroma protein from beef muscle. The preventive action of cystine supplementation with these diets with some groups of larvae and not with others suggested different margins between the cystine need and the supply. More

cystine may have been lost in the later muscle extractions, or the animals fed these materials may have had less cystine in their preliminary feeding. Possibly some additional substance needed for lens health was lost during the later extractions. Curtis, Hauge and Kraybill ('32) found that "a white opaqueness of the eye . . . . quite different from ophthalmia due to vitamin deficiency" appeared in rats fed a diet with inadequate protein when it was supplemented by cystine plus tyrosine, but did not appear when the diet was supplemented by cystine plus tryptophane. Recently, Totter and Day ('41) have produced cataracts in rats fed a diet deficient in tryptophane but supplemented with cystine. The effect of this diet supplemented with tryptophane and not with cystine has not been shown. Few data have been published on the amino acid content of the muscle proteins. According to Bailey ('37), myosin does not contain a significantly small amount of tryptophane, but its content of cystine is low.

Studies of the second type of cataract produced in the salamander larvae offer definite returns because of the ease and regularity with which the early stage can be obtained and because of the slow progress of the disease. Curative attempts may be made as well as an experimental analysis of suppressing and preventive factors. The relation between this type of the lens disease and the general health of the animals has yet to be determined.

The lens is a structure peculiarly susceptible to dietary influence because of its continued growth throughout life (Bourne, '37). For this reason, the experimental production of cataracts in growing animals and the development of cataracts in adults are not without some similarities. Moreover, the fundamental metabolic processes of the lower and higher animal forms are the same. The small carnivorous salamander larvae with their low total food intake and their high protein requirements are especially suitable for the study of problems of protein metabolism, as in the changing lens.

SUMMARY

Two types of cortical cataract have been produced in larvae of the tiger salamander, Amblystoma tigrinum, by three series of synthetic diets differing in their chief constituent, protein. Eyes were normal in larvae fed raw muscle or liver or artificial diets containing these tissues as chief ingredients.

Cataracts rarely visible in an early stage as radiating striae, with swelling followed by extensive shrinking, were produced in 43% of the animals on diets of purified casein and powdered milk and in 51% on diets containing separate muscle proteins. Preparation of each of these materials involved the use of dilute alkali, known to disintegrate cystine. The quantities of this amino acid used as supplements resulted in prevention of the lens disease in larvae fed the casein diet and in two groups of those fed the muscle-protein diets. Riboflavin was not a preventative.

Cataracts with an always visible early line of zoning parallel and near to the lens surface appeared in larvae fed high hemoglobin rations. The stage of whitening, which occurred in 37% of the animals, was accompanied by a large fluid content within the lens capsule and subsequent changes were slow. Prevention was not attained with riboflavin nor with a high supplement of cystine alone. A combination of the three amino acids of glutathione reduced the early symptoms of the disease and halted its development, but did not bring about complete prevention.

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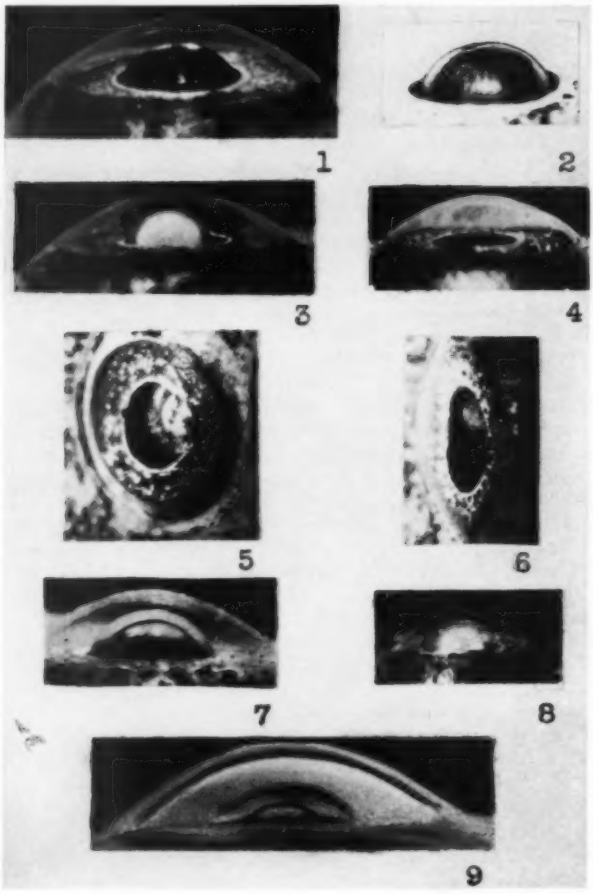

1 Normal lens by reflected light. × 10.
2 Early stage of cataract caused by diet of purified casein. Striae between equator and anterior pole of the lens. × 20.
3 Stage of maximum swelling. × 10.
4 Depressed pupil after shrinking of lens. × 10.
5 Stage of swelling in case of subluxation. × 10.
6 Subluxated lens, 39 days later, during shrinking process. × 10.
7 Dark line between transparent outer and inner layers of normal lens tissue. × 20.
8 Stage of swelling in cataract of type II. × 10.
9 Wide outer band of normal lens tissue formed after transfer of animal to diet of raw liver. × 20.

The photographs were made with equipment of the Department of Anatomy, University of Wisconsin Medical School, under the direction and with the help of Prof. H. W. Mossman.

THE INFLUENCE OF PREVIOUS REGIMES OF PROTEIN FEEDING ON THE ENDOGENOUS NITROGEN METABOLISM OF RATS

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ONE FIGURE

(Received for publication June 16, 1941)

In the determination of biological value of proteins by the method of nitrogen balance the most important question concerns the length of time the animals must be kept on a nitrogen-free diet to assure that the nitrogen excretion may reasonably be called endogenous only. Since the early work of Voit (1866) it has been axiomatic that the higher the level of protein on which an animal is living, the longer the time required to rid the organism of "circulating" protein. To secure uniformity of results Hindhede ('26) rightly insisted upon the necessity of an adequate preliminary period. Under ordinary conditions in rats this interval has been accepted as 1 to 4 days. Mitchell and his co-workers ('22, '23–'24) found that a rapid excretion of nitrogen occurred during the first 2 days of a protein-free diet, after which in some cases, an approximate constancy of excretion obtained; these results were confirmed by others (Mason and Palmer, '35; Chick et al., '35) on adult male rats. On the other hand, Ashworth and Brody ('33) found that with growing rats constancy might not be attained in less than 3 to 4 weeks. When a diet containing 4% of egg protein is used in place of the nitrogen-free diet as a conditioning ration (Mitchell and Carman, '26), the deprivation of protein is less, and appetite and body weight should be better maintained, but any periods of realimentation involve protein storage

since the experimental diets are of necessity always low in protein. The status of this and of other aspects of the subject was reviewed by Boas-Fixsen ('35).

The question of the true endogenous level became of particular importance in connection with a report of the biological value of bread proteins (French and Mattill, '35) which contained data and conclusions from experiments on human subjects and on young and adult rats. The figures from adolescent rats were considered unsatisfactory because nitrogen excretion on the nitrogen-free diet failed to become stationary even after 10 days. It seemed possible that in such animals an exceptional amount of circulating protein might be a normal accompaniment of physiological development. This appears to be only partially true; the conditions responsible for the abnormally high protein reserve were again applied, this time purposely, and the same results were obtained, all of which are here briefly described.

In the original study the biological values were measured on the same lot of about thirty rats during the three stages of growth mentioned. In the experimental periods the animals were on protein-free diets and on bread diets containing 5% of protein. For realimentation between these periods they were given a complete diet, ad libitum, until they reached the next stage. The clue to what had happened appeared when the observations on maturing animals were repeated several years later, some of the animals having gone through a similar balance study during adolescence, others being taken directly from stock.

EXPERIMENTAL

The percentage composition of the nitrogen-free diet was as follows: corn starch 77, salts 4, cellu flour 2, hydrogenated vegetable oil 15, cod liver oil 2. This diet contained 0.05% nitrogen. One vitamin [1] tablet or its equivalent was fed daily; it supplied 5–7 mg. of nitrogen. The animals were kept in wire cages, ¼ inch mesh, and the urine was absorbed on nitrogen-free filter paper prepared by moistening with an

[1] Harris, from the Harris Laboratories, Tuckahoe, New York.

alcoholic solution of boric acid and drying. At the end of each 2-day period the feces were removed for analysis and the paper was reduced to pulp by mechanical stirring in dilute acid solution, filtered on a Buchner funnel and filtrate and washings made to volume from which aliquots were taken for analysis (Kjeldahl).

The average course of urinary nitrogen excretion of all the animals (in milligrams per 100 gm. body weight) is given in the chart. A preceding low protein diet showed its effect in one group of the young animals (C). The addition of milk and lettuce to the dog-biscuit stock diet (A) also showed its effect, since even after 12 days the nitrogen output had not become constant; in animals (B) taken directly from stock diet urinary nitrogen became relatively constant by the eighth day. The differences between these three groups are such as would be expected.

Of the adolescent animals, those coming directly from stock diet and having no previous experimental history (E) had a relatively constant nitrogen output after the fourth day. Surprisingly, those (D) which had undergone realimentation for 3 weeks immediately succeeding a 3-week period on non-protein and low protein diets had not arrived at a constant output even after 10 days. Unfortunately the analytical data were not available soon enough to demonstrate the necessity of prolonging the non-protein feeding period.

In contrast, the grown animals came to relatively constant excretion in 4 to 6 days, irrespective of whether they had 2 months previously suffered from brief protein starvation (H) or were taken directly from stock diet (G). The group of ten slightly smaller and younger animals (F) (a part of lot E) had been on ad libitum stock diet for 2 weeks following a period of 18 days on nitrogen-free diet. At the end of 4 days their nitrogen excretion had fallen to a relatively low level, but this rose again and did not decline to the 4-day level of groups G and H until after 16 days. Figures for fecal nitrogen obtained throughout these studies parallel those for urinary nitrogen, but with much smaller fluctuations.

The interpretation of the behavior of the young rats is obvious; their endogenous nitrogen was related to the previous dietary nitrogen. The adolescent animals (D) had been deprived of protein during a period of rapid development. In the 3 weeks of ad libitum feeding they more than compensated for the loss and had stored an excess of protein much above that which they would normally have accumulated (E). How long a period would have been required for a reduction of this excess to normal levels is uncertain. In adult animals (H), a 2 months period following brief protein deprivation was sufficient to permit this excess to be lost. Furthermore their demands for protein were largely for maintenance and less

EXPLANATION OF CHART

Excretion of urinary nitrogen (mg. per 100 gm. body weight) by rats on N-poor diet following diets with various levels of nitrogen intake.

GROUP	PREVIOUS DIETARY HISTORY	NUMBER OF ANIMALS	BODY WEIGHTS DURING N-FREE PERIOD		LENGTH OF PERIOD	FRACTION OF ORIGINAL WEIGHT LOST
			Beginning	End		
			gm.	*gm.*	*days*	%
A	Dog biscuit, milk, lettuce	10 ♀	58 (45–74)	42	12	28
B	Dog biscuit	8 ♂, 8 ♀	50 (43–64)	36	18	28
C	3 weeks on low-protein	31 ♀	62 (45–80)	50	10	20
D	Dog biscuit for 3 weeks, following 20 days of non-protein (10) and low protein (10) diet	31 ♀	136 (90–158)	97	10	29
E	Dog biscuit	7 ♂, 12 ♀	126 (95–147)	99	18	21
F	Dog biscuit for 2 weeks following 18 days on N-free diet	6 ♂, 4 ♀	181 (170–211)	131	18	28
G	Dog biscuit	6 ♀	215 (202–224)	174	18	19
H	Dog biscuit for 2 months following several periods of low- and non-protein diets	31 ♀	184 (151–211)	169	12	8

for growth, the urge was less and the smaller shortage was restored more quickly. Losses in body weight in the nitrogen-free period were greater when realimentation immediately preceded this period (compare D and F with E and G); they were also greater in young animals (A, B) than in older animals (G, H).

The contrast in behavior between groups F and G is particularly disturbing because many studies of biological value have been made on animals within this weight range. The previous deprivation of protein suffered by the animals in group

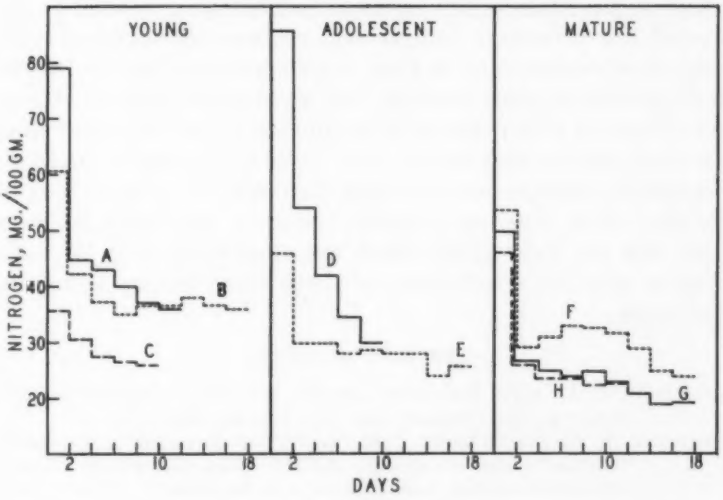

Chart 1

F may well explain their higher nitrogen output after 2 weeks of ad libitum feeding, as compared with the nitrogen excretion of the G animals, but it does not explain their subsequent increased and continued higher level of nitrogen output, while that of the G animals again began to decline after 10 days on the nitrogen-free diet. Even after ad libitum stock feeding without any experimentation, endogenous nitrogen excretion sometimes takes this course (Ashworth, '35) in maturing but

not in young animals. Obviously, the use of short non-protein feeding periods would lead to quite different figures for the biological value of proteins fed to animals like those in the F and G groups.

Many of the studies on biological value may have been complicated by this unforeseen departure from the ordinary course of endogenous nitrogen excretion.

SUMMARY

When rats employed in the determination of biological value of proteins by the balance method are subjected to repeated low protein or non-protein regimes interspersed with periods of realimentation they overcompensate for the loss of body protein in these periods, and accumulate unusual stores. As compared with controls such animals require a longer time on a low protein diet before their urinary nitrogen falls to an acceptable endogenous level and also suffer a greater loss in weight. This was particularly noted in maturing animals (100–200 gm. in weight) which are commonly used for such studies and the significance of these observations is briefly discussed.

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OBSERVATIONS ON INDUCED CARIES IN RATS

III. EFFECT OF FLUORIDE ON RAT CARIES AND ON COMPOSITION OF RATS' TEETH

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TWO FIGURES

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Epidemiological studies relate a partial prevention of human dental caries to the occurrence of fluorides in drinking water (Dean, '38; Dean, Jay, Arnold, McClure and Elvove, '39; Dean, Jay, Arnold and Elvove, '41 a). Induced dental caries in rats also is inhibited by the ingestion of fluoride (Miller, '38; Finn and Hodge, '38; McClure and Arnold, '41). These effects may be due to: (a) inhibition of bacterial action on food residues (Lundsgaard, '30), or dental tissue (Robison and Rosenheim, '34); (b) a systemic effect of fluoride on tooth formation (Armstrong and Brekhus, '38; Cox, Matuschak, Dixon, Dodds and Walker, '39; Volker, '39; Dean, Jay, Arnold and Elvove, '41 b), or (c) a modified saliva (Cheyne, '40; Wills, '40; McClure, '41).

Enamel may become hypoplastic (mottled) as a result of fluoride ingestion during the period of dental calcification. Fluoride has no known post-eruptive effect on human dental enamel, although certain data (Perry and Armstrong, '41) indicate that in rats the oral enamel surface may adsorb fluorides from drinking water. It remains to be demonstrated that post-eruptive ingestion, or a local oral treatment with fluoride, will add significant quantities of fluorine to human enamel, or by some other action inhibit human dental caries.

[1] From the Division of Infectious Diseases, in cooperation with the Division of Chemistry and the Division of Chemotherapy.

EXPERIMENTAL

The experimental plan follows a previous study (McClure and Arnold, '41). Groups of rats aged 23–25 days, sixteen to twenty-three litter mates in control and corresponding test groups, were on a test period of 15 weeks. A cornmeal caries-producing diet was fed to all groups, ad libitum. The caries effect of this diet is generally attributed to the size of the corn particle (Lilly and Wiley, '34). The diets as used averaged 0.3–0.5 ppm. fluorine. Except in two test groups fluoride was added via drinking water. Water intake was not controlled, the results being based, therefore, on fluoride concentration of water or food, and on fluoride given by subcutaneous injection. Comparisons are made between a test group and its corresponding control (McClure and Arnold, '41).

After examining the molar teeth for occlusal caries (Cox, Dodds, Dixon and Matuschak, '39), pooled samples of the crowns of the molars and the erupted part of the incisors of the various groups were pulverized to pass a 60-mesh sieve, the dentine and enamel separated (Manley and Hodge, '39), and fluorine (McClure, '39 a) and ash determined. Pooled samples of the combined dentine and enamel of carious and non-carious rats' molars were analyzed for ash, calcium, phosphorus, and fluorine. Certain of the data was obtained from other experiments (McClure and Arnold, '41; Arnold and McClure, '41).

RESULTS

Fluorides equalling 50 and 100 ppm. fluorine in water, and 125 ppm. in food, inhibit rats caries decisively (table 1 and fig. 1). It may be concluded that 10 ppm. fluorine in water also inhibited caries as studied. Thus, in fifty-six possible comparisons between litter mate control and test rats, the 10 ppm. fluorine rat showed a lower caries score in thirty-eight instances. The odds are 1 to 259 against this being a fortuitous happening (Student, '25). Rats ingesting 5 ppm. fluorine in water were not different from their controls. The effect of injected fluorine on induced rat caries appears to be negative,

TABLE 1

Effect of fluoride on rat caries.

TREATMENT	NUMBER OF RATS PER GROUP	RATS WITH CARIES	CARIOUS MOLAR TEETH	CARIOUS MOLAR TOOTH AREAS	AVERAGE CARIES SCORE[1]	
					All rats	Carious rats
		per cent	*per cent*	*per cent*		
Control A	23	95.6	27.5	20.6	17.1	17.9
10 ppm. F. in water	23	69.6	14.9	10.5	8.2	11.8
Control B	27	100.0	35.2	31.4	27.6	27.6
10 ppm. F. in water	24	79.2	26.7	18.8	15.6	19.7
100 ppm. F. in water	26	57.7	13.8	7.8	6.2	16.1
Control C	26	80.8	24.4	20.6	18.0	22.3
5 ppm. F. in water	25	88.0	24.3	20.0	17.9	20.3
50 ppm. F. in water	25	52.0	10.7	5.5	4.0	7.6
Control D	25	76.0	24.7	15.8	10.6	13.9
125 ppm. F. in food	25	12.0	1.0	0.4	0.2	1.3
Control E	23	100.0	27.9	19.4	15.7	15.7
10 ppm. F. in water	20	80.0	22.1	16.6	14.0	17.4
NaF injected	24	83.0	22.2	18.8	16.2	19.4

[1] There are a total of thirty-four areas distributed over the twelve molar teeth in which areas cavities are most likely to occur. Depending on its size, each cavity is given a score of 1, 2, or 3 (see Cox, Dodds, Dixon and Matuschak, '39).

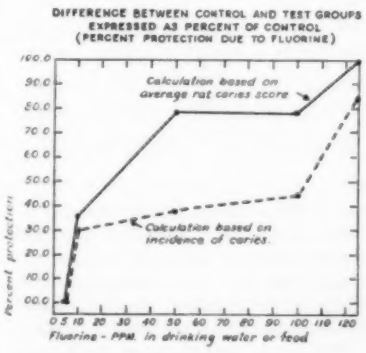
DIFFERENCE BETWEEN CONTROL AND TEST GROUPS EXPRESSED AS PERCENT OF CONTROL (PERCENT PROTECTION DUE TO FLUORINE)
Calculation based on average rat caries score
Calculation based on incidence of caries
Percent protection
Fluorine - PPM. in drinking water or food

Figure 1

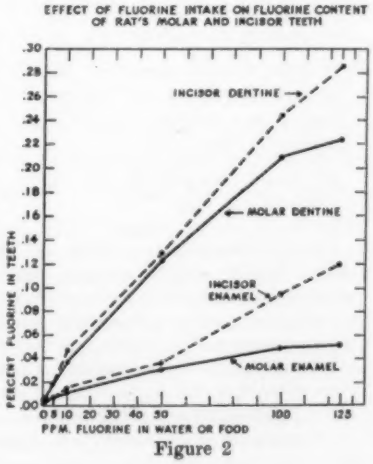
EFFECT OF FLUORINE INTAKE ON FLUORINE CONTENT OF RAT'S MOLAR AND INCISOR TEETH
INCISOR DENTINE
MOLAR DENTINE
INCISOR ENAMEL
MOLAR ENAMEL
PERCENT FLUORINE IN TEETH
PPM. FLUORINE IN WATER OR FOOD

Figure 2

although these rats received an average daily intake of fluorine approximately equal to 50 ppm. of fluorine in drinking water.[2]

The ash of molar and incisor dentine and enamel was not affected by fluoride. Similarly there was no difference in ash, calcium, phosphorus, and fluorine of mixed dentine and enamel of molars of carious rats, as compared with non-carious rats (table 2).[3]

TABLE 2

Ash, calcium, phosphorus, and fluorine in molar teeth of carious and non-carious rats.

TREATMENT	NUMBER OF RATS	AVERAGE CARIES SCORE	COMPOSITION OF MIXED DENTINE AND ENAMEL			
			Ash	Ca	P	F
			per cent	*per cent*	*per cent*	*per cent*
Control A non-carious	5	0.0	78.2	29.1	15.1	.0031
Control A carious	17	17.9	77.0	28.9	14.7	.0035
10 ppm. F. non-carious	10	0.0	77.7	28.9	14.8	.0297
10 ppm. F. carious	13	11.8	75.3	27.7	14.1	.0291
Control B carious	27	27.6	76.9	28.9	14.4	.0032
10 ppm. F. non-carious	6	0.0	78.3	28.7	14.7	.0339
10 ppm. F. carious	18	19.7	77.5	28.5	14.9	.0365
100 ppm. F. non-carious	12	0.0	77.4	29.4	14.5	.1866
100 ppm. F. carious	14	16.1	77.4	29.1	14.8	.2009
Control C non-carious	5	0.0	79.1	28.8	15.4	.0046
Control C carious	21	22.3	77.5	28.5	14.8	.0035
5 ppm. F. non-carious	4	0.0	79.8	29.4	15.2	.0192
5 ppm. F. carious	21	20.3	76.8	28.6	14.5	.0245
50 ppm. F. non-carious	13	0.0	77.4	29.1	14.9	.1140
50 ppm. F. carious	12	7.6	78.3	29.1	14.9	.1069
Control D non-carious	8	0.0	79.2	29.8	14.9	.0025
Control D carious	17	13.9	78.3	29.7	14.6	.0030
125 ppm. F.[1] non-carious	22	0.0	78.9	29.7	15.0	.1842

[1] Fluoride in food.

[2] These injection rats (Arnold and McClure, '41) received 0.5 mg. fluorine as sodium fluoride injected subcutaneously every other day for the first 24 days and every day thereafter for the remaining experimental 75–76 days. At the start of the regular injection period, these rats averaged 41.3 days old, as compared with their controls aged 32.8 days, a delay caused by a local irritation which developed at the site of the first injection. An age factor therefore might have influenced these older rats slightly in caries response. This group of rats, however, did not differ significantly from the controls.

[3] Samples of mixed dentine and enamel, as prepared from carious teeth, may have contained a less than normal quantity of enamel as a result of their carious condition. This would tend to decrease the ash results abnormally on these samples.

DISCUSSION

The course of prevention by fluoride of induced rat caries (fig. 1) appears to support an anti-enzymatic mode of action. Inhibition occurred at the low level of 10 ppm. of fluorine; it was equally pronounced at 50 ppm. and 100 ppm., and was practically complete at 125 ppm. Since the observed action was not similarly proportional to fluoride deposited in the teeth, there is the suggestion of an effect on reactions occurring locally within the oral cavity. In this connection also, it may be noted that comparison of the two curves (fig. 1) indicates fluoride was perhaps less effective in preventing the initiation of caries (caries incidence) than in retarding its development (caries score).

On the basis of indirect evidence, the etiology of rat caries itself appears to involve an enzyme action within the oral cavity. Thus iodoacetic acid, which, like fluoride, is an active anti-enzymatic substance, inhibits rat caries (Miller, '38; McClure and Arnold, '41). Likewise fluoride which does not reach the oral cavity, i.e., when given by injection, is not caries-inhibitory (Arnold and McClure, '41). In conjunction with the indicated anti-enzymatic effects of fluoride, a bactericidal effect on oral flora is not eliminated.

Results of fluorine analysis of the rats' teeth demonstrate a considerable retention of fluorine by molar dentine and enamel as occurring after tooth eruption (fig. 2). In regard to the rat's incisors, which are continuously erupting, deposition of fluorine is not unusual (McClure, '39 b). However, the similarity of the added quantities of incisor fluoride and molar fluoride [4] particularly for 5, 10, and 50 ppm. rats,

[4] The rat's first and second molar teeth are fully erupted by the twenty-fifth day; the third molars are fully formed at this time, but are not erupted until about the thirtieth day. The apposition of primary dentine in rats' molars (Hoffman and Schour, '40) may possibly account for a part of the fluorine increment in molar dentine. Molar enamel growth, however, is presumably complete at about 10 days of age (Hoffman and Schour, '40). A post-eruptive enlargement of rat molars as reported by Donaldson ('34) was not confirmed by Wood ('35) nor by Hoffman and Schour ('40).

As was to be expected, the rats' incisor teeth showed the effects of fluorosis. The molar teeth were normal in appearance.

implies the acquisition of fluorine by incisors following the developmental stages.

It is particularly significant in these regards that the molar enamel of fluoride injected rats averaged 0.0131% fluorine as compared with 0.0034% fluorine for controls (Arnold and McClure, '41). Since it is doubtful that this increase in enamel fluoride was due to adsorption from saliva (McClure, '41; Willis, '40), these data seemingly indicate that the erupted dentine and enamel retained fluoride from the systemic circulation. Other evidence in the data supports this belief. Thus, the ratio of fluorine in dentine and enamel for molars and incisors is as follows:

Fluoride treatment (ppm.)	10	10	10	50	100	125
Molar teeth (ratio)	3.74	3.23	3.23	3.96	4.20	4.31
Incisor teeth (ratio)	2.52	3.43	3.11	3.47	2.59	2.38

This relatively constant proportion of fluorine deposited in these two dental tissues should doubtless be ascribed to a regular systemic retention, which is seemingly unaffected by oral surface adsorption of fluoride.

The property of the oral enamel surface as an adsorbent of water-borne fluoride has been tested recently by Perry and Armstrong ('41). Their data give some evidence that enamel has this property. Contrary to the findings of this report, however, Perry and Armstrong ('41) found no increase in fluorine in erupted rat molar dentine. Perhaps the age of their rats (mature male rats averaging 250 gm.) and the duration of their experiment (60 days) may account for the different results obtained.

Post-eruptive deposits of fluorine may not increase the tooth's caries resistance. Thus, although fluorine increased in the molars of injected rats, caries remained the same as in control rats. A similar result applies to rats receiving 5 ppm. of fluorine in water. Also the fluorine in the molars of three groups of rats given 10 ppm. fluorine water is not proportional to the observed caries prevention. At the present time, therefore, it seems unlikely that these secondary post-eruptive

deposits of fluorine exerted a significant effect on the development of rat caries. Except for the injection group, perhaps any effect of tooth fluoride may have been masked by food or water-borne fluoride.

SUMMARY

A minimum of 10 ppm. of fluorine in drinking water gave partial protection against occlusal rat caries. Approximately 80% protection resulted from both 50 ppm. and 100 ppm. fluorine in water. It is suggested that fluorine acts to inhibit rat caries by anti-enzymatic local action within the oral cavity.

The pooled molar teeth of groups of carious rats were not different from those of non-carious rats in content of ash, calcium, and phosphorus, and in fluorine which was deposited post-eruptively.

The post-eruptive addition of fluorine to rats' molar teeth occurred in relatively large quantities and is regarded as occurring independent of oral enamel surface adsorption of fluoride. A significant effect of fluoride acquired after tooth eruption on induced rat caries was not evident.

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STUDIES ON THE RAT GROWTH ASSAY METHOD FOR RIBOFLAVIN

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ONE FIGURE

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The recent status of the rat growth procedure for the assay of riboflavin was adequately summarized by Wagner, Axelrod, Lipton and Elvehjem ('40). This report indicates that the older diets, such as the widely used Bourquin-Sherman ('31) ration, appear to be inadequate in certain factors necessary for optimal nutrition of the rat, exclusive of riboflavin. More recently diets have been described which support nearly normal growth when supplemented only with riboflavin. Some of the materials that have been proposed to supply the vitamin B complex in these diets are charcoal-treated liver extract (El Sadr, Macrae and Work, '40), fuller's earth-treated rice bran extract (Clarke, Lechycka and Cook, '40), fuller's earth-treated yeast extract (Hunt and Bethke, '40), and photolyzed liver concentrate plus thiamine and vitamin B_6 (Wagner, Axelrod, Lipton and Elvehjem, '40).

In the author's experience, an untreated rice bran extract has been found to be a satisfactory source of the B vitamins, other than riboflavin, needed by the rat and thus suitable for the preparation of a standard riboflavin-low basal diet. Extensive experiments involving more than 1500 rats have shown that the ration proposed permits a high degree of assay accuracy. It is believed that the diet thus developed offers certain distinct advantages as to simplicity and ease of preparation over other riboflavin-deficient diets that have been described.

EXPERIMENTAL

Young male rats from our laboratory colony, weighing 40 to 55 gm., are placed in individual wire cages in an air-conditioned room, the temperature of which is kept at 77° ± 2°F. The diet used is a modification of that described by Street and Reeves ('40) and consists of a vitamin B complex-free basal mixture fed ad libitum, supplemented at standard time intervals with rice bran extract. The basal mixture has the following percentage composition: vitamin-free casein [1] 18; cornstarch 63; hydrogenated cottonseed oil [2] 12; cod liver oil 3; and salt mixture (no. 2 of U. S. P. XI) 4. In addition to this ration, each rat is given an aqueous extract of rice bran [3] three times weekly, fed in small supplement dishes, at a level supplying 15 μg. of thiamine per rat daily. This level of supplement has proved adequate for test purposes, since with the addition of optimal amounts of riboflavin it permits a very satisfactory, although slightly subnormal, growth rate on the basis of data from our colony (fig. 1). However, it might be advisable for very rapidly growing strains of rats to increase somewhat the amount of the extract given. In place of the above supplement one may use with equal success the 25% alcoholic extract of rice bran described by Street and Cowgill ('39) at a level equivalent to 1 gm. rice bran per rat daily, as indicated by Street and Reeves ('40).

The animals are weighed twice weekly starting 1 to 2 weeks after the beginning of the depletion period. They are considered ready for testing when the weight gains approximate 4 gm. or less in the last 10 days; but animals gaining up to 6 gm. in 10 days are also considered satisfactory for assay purposes if they have not gained more than 2 gm. during the last week. The depletion period on the above diet at

[1] Labeo brand.

[2] Crisco.

[3] Vitab Rice Bran Concentrate, obtainable from the National Oil Products Company, Inc., Harrison, New Jersey. This material "is an aqueous extract of rice bran concentrated to a specific gravity of 1.38 and clarified by filtration." The vitamin content of this preparation is constant over long periods of time when it is stored under refrigerator conditions.

certain seasons of the year has been found to be 2 to 4 weeks; at other seasons, 3 to 5 weeks, as indicated in table 2. There are usually a few animals, never more than 10% of the total, which continue to gain after the weights of the others have reached a plateau. These exceptional animals are discarded. Test groups of ten or more animals are used for both assay and control purposes. Three control groups are used for each assay and include a group of negative controls to which no riboflavin is given, a second group with a supplement of 5 μg. of crystalline riboflavin per day, and a third group which receives 10 μg. of riboflavin per day. The 7-day dosage supplement is given on a 6-day basis, i.e., each day-dose represents one-sixth the amount consumed weekly. However, experience has indicated that the test fractions may be given three times weekly in twice the calculated day-dose. This sequence, as recommended by Carlsson and Sherman ('38), is entirely satisfactory and economical as to time. The standard solution of riboflavin or the preparations to be assayed are measured into individual supplement dishes containing the basal rice bran extract supplement.

The test and control animals are weighed weekly during the period of assay and the average gain in weight for this 4-week period is calculated for each group. The growth of the negative control animals on the basal ration alone averages 6 to 9 gm. in 4 weeks, but the maximal range is 0 to 12 gm. The growth induced by various riboflavin dosage levels under the above test conditions is shown in table 1.

The results have been expressed in terms of the effective gain, that is, the gain of any group minus the gain of the negative controls. It may be noted that, at a given season of the year, the weight gain per unit dosage of riboflavin is the same within the limits of 2.5 and 10 μg. of the vitamin per day.

The growth response to varying amounts of riboflavin throughout a 6-week period following depletion is shown graphically in figure 1. The gain of 70 to 80 gm. in 4 weeks induced by a supplement of 50 μg. of riboflavin daily ap-

proaches the normal growth rate, since the rats of this colony usually gain about 100 gm. in 4 weeks on a stock ration.

In calculating the results of an assay the effective gain per microgram of riboflavin is obtained from the performance of the control groups at the 5 and 10 μg. levels, and from this figure the riboflavin content of the day-dose of the unknowns is calculated. These procedures permit an evaluation of unknown substances administered at a single dose level, providing the actual content of the day-dose lies between 2.5 and 10 μg. (The response at higher levels, although materially greater, has not been investigated on a quantitative basis.)

TABLE 1

The rate of growth induced by supplementing the basal diet with various amounts of riboflavin.

ASSAY NO.	DATE BEGUN	NO. OF RATS USED	DAILY DOSE OF RIBOFLAVIN	EFFECTIVE GAIN IN 4 WEEKS [1]	GAIN PER MICROGRAM OF RIBOFLAVIN
			μg.	*gm.*	*gm.*
		Assays conducted in 1939			
4	May	9	6.25	27.5	4.4
		10	7.50	29.3	3.9
		10	8.75	36.4	4.2
5	June	9	7.5	34.2	4.6
6	June	10	5.0	20.1	4.0
		9	7.5	28.3	3.8
		10	10.0	42.1	4.2
		Assays conducted in 1940			
14	January	10	2.5	8.1	3.2
		11	5.0	18.2	3.6
15	March	10	5.0	17.6	3.5
		10	10.0	33.8	3.4
16	April	11	5.0	18.6	3.7
		11	10.0	37.7	3.8
17	May	10	5.0	20.9	4.2
		10	10.0	41.9	4.2
18	June	9	5.0	22.6	4.5
		9	10.0	44.8	4.5
19	July	9	2.5	11.3	4.5

[1] The effective gain indicates the total gain in weight of any group minus the gain of the negative controls.

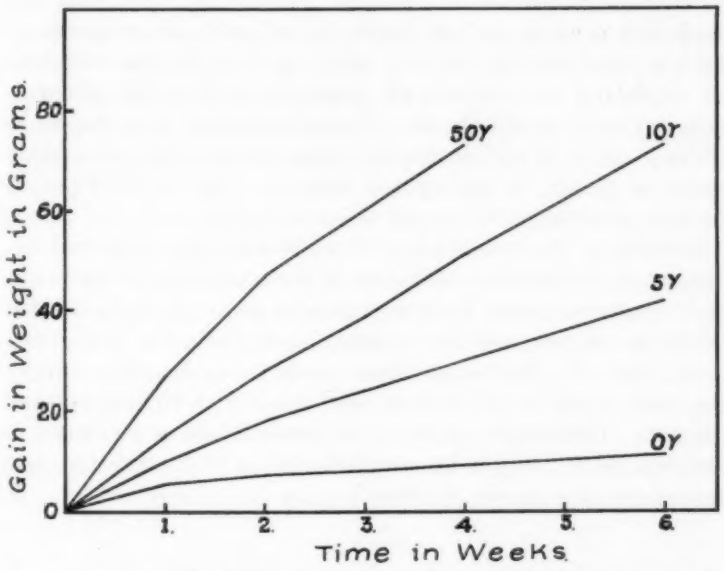

Fig. 1 Graph showing the growth induced by supplementing the basal diet with various levels of riboflavin. The figure above each curve indicates the daily dose of riboflavin in micrograms.

TABLE 2

Seasonal variation in depletion period.

ASSAY NO.[1]	DATE BEGUN	MEAN LENGTH OF DEPLETION PERIOD	ASSAY NO.[1]	DATE BEGUN	MEAN LENGTH OF DEPLETION PERIOD
	1939			1940	
		days			*days*
4	May	34.5	13	January	18.5
5	June	34.6	14	January	16.6
6	June	31.0	15	March	17.5
7	July	33.5	16	April	17.9
8	August	31.4	17	May	20.8
9	September	26.4	18	June	23.5
10	October	21.9	19	July	24.8
11	November	19.2	20	September	21.9
12	December	19.9	21	November	18.0
			22	December	17.0

[1] Each assay represents sixty to eighty male rats.

Seasonal variations in results. Despite the fact that the stock diet used in our rat colony is of uniform composition, there is a definite seasonal variation both in the time required for depleting the animals of riboflavin and in the effective gain per unit of riboflavin. These variations are shown in tables 1 and 2. It will be observed that the necessary depletion period is longer in spring and summer. During this period the animals also make larger gains in weight.

Validity of the procedure. It is obvious that a partial deficiency of the basal or test diets in any essential factor other than riboflavin would lead to a greater gain per unit of riboflavin, as represented by natural foods, than for pure riboflavin, since the food supplement could be expected to supply the other factors present in the basal diet in sub-optimal amounts. The results of assays of several lots of a vitamin B complex elixir, added as supplements to the suggested test procedures, are shown in table 3.

TABLE 3

Riboflavin assays of vitamin B complex elixir.

SAMPLE NO.	ACTUAL RIBOFLAVIN CONTENT	RIBOFLAVIN CONTENT FOUND BY ASSAY	
		Absolute content	Per cent of true value
	μg./cc.	*μg./cc.*	%
1	25	24.5	98
2	25	26.5	106
3	25	27.8	111
4	25	25.3	101
5	25	25.8	103
6	25	27.2	109
7	25	24.8	99
8	25	27.2	109
9	25	24.2	97
10	25	29.8	119
11	25	25.0	100

In this preparation the riboflavin content has been fortified by means of the synthetic material, so that only 20% of the total present is represented by the natural riboflavin contained in rice bran extract. Thus, the actual riboflavin content is known with considerable exactness. It will be observed that

the method applied to this material shows approximately the expected content, indicating that the various water-soluble vitamins other than riboflavin present in rice bran extract do not appreciably affect the growth rate.

As a further check on the validity of the method assays were made with a preparation of known riboflavin content. Filtrate factor free of riboflavin and of vitamins B_1 and B_6 was prepared by treating rice bran extract five times with fuller's earth at pH 4.0. This filtrate factor was then fortified with synthetic vitamins in such amounts that the final preparation contained 10 μg. of riboflavin, 30 μg. of thiamine, and 25 μg. of vitamin B_6 per cubic centimeter. Another preparation containing approximately 10 μg. of riboflavin per cubic centimeter was prepared by adding a solution of riboflavin to rice bran extract, so that the final solution contained per cubic centimeter 2 μg. of riboflavin from the extract and 8 μg. of synthetic riboflavin. The results of assays of these two preparations are shown in table 4.

TABLE 4

Assay of complex preparations containing 10 μg. of riboflavin per cubic centimeter.

GROUP NO.	SUPPLEMENT	ACTUAL RIBOFLAVIN CONTENT OF DAILY DOSE	AVERAGE WEIGHT GAIN IN 4 WEEKS		APPARENT RIBOFLAVIN CONTENT OF DAILY DOSE
			Total gain	Gain over negative controls	
		μg.	gm.	gm.	μg.
1	None	0	7.7		
2	Riboflavin	2.5	19.0	11.3	
3	Rice bran extract plus riboflavin	5.0	33.4	25.7	5.7
4	Filtrate factor plus riboflavin, thiamine, and vitamin B_6	5.0	31.7	24.0	5.3

The effective gain (growth of assay group minus growth of negative controls) of 11.3 gm. in 4 weeks for the control group receiving a 2.5 μg. dose of riboflavin is equivalent to 4.52 gm. per microgram of riboflavin. From this figure the apparent riboflavin content of the daily dose has been calculated for groups 3 and 4. It will be seen that the observed value of the

fortified filtrate factor, the riboflavin content of which was known with exactness, was only 6% greater than the true value.

These data appear to indicate that the use of the assay procedure described permits satisfactory determinations of the riboflavin contained in natural products.

Experiments with wheat extract as a source of the vitamin B complex. Frequently large discrepancies exist between riboflavin assays of products by different laboratories. Presumably these variations are to a considerable extent due to differences in the diets used. To determine more definitely the difference that might be expected because of variation in diet, the author's procedure has been compared directly with the original Bourquin-Sherman method, which utilizes an 80% alcoholic extract of wheat as the source of the vitamin B complex of the diet. The result of the assay of substances of known riboflavin content by this procedure is shown in table 5.

TABLE 5

Experiments with a ration containing wheat extract as a source of the vitamin B complex.

GROUP NO.	SUPPLEMENT	ACTUAL RIBOFLAVIN CONTENT OF DAILY DOSE	AVERAGE WEIGHT GAIN IN 3 WEEKS	
			Total gain	Gain over negative controls
		μg.	*gm.*	*gm.*
1	None	0	—3.7	
2	Synthetic riboflavin	2.5	2.8	6.5
3	Synthetic riboflavin	5.0	5.7	9.4
4	Synthetic riboflavin	10.0	9.0	12.7
5	Vitamin B complex elixir, sample 2	2.5	15.8	19.5
6	Filtrate factor plus riboflavin, thiamine, and vitamin B_6	2.5	17.4	21.1
7	English ale yeast	2.5	12.6	16.3

The elixir and the fortified filtrate factor correspond to those previously tested (tables 3 and 4). The value expressed for the English ale yeast was obtained by the author's assay procedure. The data presented have been calculated on the basis of the 3-week gains since the flattening of the growth curve mentioned by Sherman and Smith ('31) appeared after the third week in this experiment.

It may be observed that the gain per unit of riboflavin was very much greater for the preparations tested than for the pure vitamin. Thus, for groups 5, 6 and 7 receiving 2.5 μg. of riboflavin per day in preparations containing natural products, the effective gain was two to three times that for group 2, which received 2.5 μg. of synthetic riboflavin per day. These results indicate clearly that it is unsafe to conclude that a particular lot of a diet may be safely used for riboflavin assay merely because it permits increasing growth with increasing amounts of riboflavin at low levels of supplementation.

DISCUSSION

The results of the experiment on the use of wheat extract are in general agreement with those of Schumaker and Heuser ('40) who found that instead of using the customary conversion factor of 2.5 μg. of riboflavin per Bourquin and Sherman unit, the true value was obtained with a conversion factor of 2.0 μg. per unit for residual yeasts and 1.3 μg. for primary grown yeasts. They took as the true riboflavin content their findings with a photometric procedure and with a chick assay procedure. Likewise Clarke and coworkers ('40) have found wheat extract to be unsatisfactory as a source of the B complex in riboflavin assays because of the presence of only suboptimal amounts of certain factors other than riboflavin.

With the diet and procedure described in this paper there is a linear relation between the growth response, expressed as the gain over the negative controls, and the dose of riboflavin administered. In contrast, Clarke and coworkers ('40) under the conditions of their procedure have found a linear relation to exist between the total gain and the logarithm of the dose administered. The author's data cannot be made to conform to linearity when similarly expressed, but the relations between dose and response are in close agreement with those presented by Sherman and Smith ('31) for the Bourquin-Sherman diet when the riboflavin is furnished by natural foods. Likewise, Hunt and Bethke ('40) using a diet containing fuller's earth-treated yeast as a source of the water-soluble vitamins, found a linear relation between the dose

of riboflavin and the growth expressed as the increase in weight over the negative controls. Similarly, Wagner, Axelrod, Lipton and Elvehjem ('40) have found that with their riboflavin deficient ration, the relation between growth and riboflavin administered is approximately linear over the range of 3 to 9 μg. of riboflavin per day.

SUMMARY

Since diets complete in all the factors required by the rat other than riboflavin have been described, there would seem to be little reason for the continued use of rations containing wheat extract, in that wheat does not appear to be an adequate source of the vitamin B complex for such purposes.

The diet and assay procedure described, in which an untreated rice bran extract is utilized for supplement purposes, represent certain improvements over available test methods and permit a rapid, accurate, and economical assay of riboflavin from either synthetic, natural or mixed (pharmaceutical) sources.

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THE NICOTINIC ACID AND COENZYME CONTENT OF THE TISSUES OF NORMAL AND BLACKTONGUE DOGS

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It has been concluded by Kohn, Klein and Dann ('39) and by Axelrod, Madden and Elvehjem ('39) that the coenzyme content of the liver and voluntary muscle of the dog is decreased in blacktongue (nicotinic acid deficiency). Neither group of investigators produced evidence which proved this point conclusively; the figures of the former authors showed too great a variation within each of the groups of dogs compared and the latter gave figures only for one normal and two blacktongue dogs. It was, therefore, decided to reinvestigate the problem more thoroughly, and in addition to the coenzyme comparison to perform parallel tissue analyses for nicotinic acid.

EXPERIMENTAL

Blacktongue was induced in a series of dogs by maintaining them on the nicotinic acid deficient regimen described by Kohn, Klein and Dann ('39), modified by altering the water-soluble vitamin supplement from 1 mg. thiamine chloride weekly to 1 mg. thiamine chloride and 1 mg. riboflavin twice weekly. The normal dogs used for comparison were kept on the stock diet described by the same authors.

The total pyridine nucleotides (coenzymes I and II) were estimated as V-factor by the method of Kohn ('38). The validity of this procedure has been confirmed by Handler and

Dann ('41) using the tissues of the rat. The tissue extracts were compared with solutions of a standard preparation of diphosphopyridine nucleotide obtained from yeast by the method of Warburg and Christian ('36). This standard was determined to be 75% pure on the basis of its nicotinic acid and phosphorus content. Nicotinic acid analyses were performed by the method of Dann and Handler ('41 a). All the dogs were decapitated for maximum exsanguination and tissue samples were taken for analysis within 15 minutes after death. The blacktongue dogs were sacrificed when they were so severely ill that death seemed certain within 24 hours.

Table 1 summarizes the nicotinic acid and coenzyme content of three tissues of ten normal dogs. The coenzyme content is expressed as micrograms of diphosphopyridine nucleotide equivalent in activity to 1 gm. of fresh tissue. This method of expression is valid because the di- and triphosphopyridine nucleotides have the same growth stimulating action on H. parainfluenzae in equimolar concentrations. By "bound"

TABLE 1

Nicotinic acid and coenzyme I and II content of tissues of dogs.

ANIMALS	FACTORS DETERMINED	LIVER			KIDNEY			MUSCLE		
		Mean & S.E.	Range	Per cent of normal	Mean & S.E.	Range	Per cent of normal	Mean & S.E.	Range	Per cent of normal
Ten normal dogs	Coenzyme I & II	438±46	290–720		504±18	388–570		310±12	250–356	
	Total NA	153±12	90–233		95± 3.4	75–117		71± 1.9	59– 80	
	Bound NA	81± 8.5	53–133		93± 3.3	72–106		57± 2.2	46– 66	
	Unbound NA	72			0			14		
Ten blacktongue dogs	Coenzyme I & II	184±19	114–309	42	500±15	416–564	100	222±28	86–360	72
	Total NA	101± 4.3	84–117	66	96± 2.7	75–104	100	59± 5.3	32– 81	83
	Bound NA	34± 3.5	21– 57		92± 2.8	77–104		41± 5.2	16– 60	
	Unbound NA	67		93	0			?[1]		?[1]
Four normal dogs with excess nicotinic acid	Coenzyme I & II	457	371–526		461	405–432		342	319–360	
	Total NA	151	142–159		91	86– 94		77	72– 86	
	Bound NA	85	70– 97		85	75– 98		64	59– 67	
	Unbound NA	66			?[1]			13		

[1] The query sign indicates that a figure could be entered by calculation from other data given, but it would have no statistical significance.

nicotinic acid is meant only that nicotinic acid bound as coenzyme; this figure is obtained on dividing the quantity of coenzyme by 5.4. By "unbound" nicotinic acid is meant all nicotinic acid other than that which is bound in coenzymes I and II.

Almost half of the nicotinic acid present in the livers of the normal dogs was found to be unbound, as was 20% of the nicotinic acid of the muscle. No unbound nicotinic acid appeared to be present in the kidney cortex. Unlike the dog, neither rat kidney cortex nor rat muscle contains any unbound nicotinic acid (Handler and Dann, '41). The statistical significance of the figures is indicated by the standard errors of the means and the ranges reported.

Table 1 also gives corresponding figures for dogs with severe blacktongue. As with the normal animals, the livers contained unbound nicotinic acid and the kidney cortex none; but the situation in muscle is uncertain. The figures given suggest that 10–20% of the nicotinic acid was unbound, as in normal dogs, but do not establish this point conclusively because the difference of the means of total and bound nicotinic acid was not significant owing to large variations within each group. About 30% of the liver nicotinic acid was unbound.

The columns in table 1 which are headed "per cent of normal" give a summary comparison of the tissues of normal and blacktongue dogs. In blacktongue the greatest change from the normal occurred in the liver; the total nicotinic acid content decreased to 66% of the normal and almost all of this decrease occurred in the bound fraction of the nicotinic acid. The coenzyme level (and, therefore, the bound nicotinic acid also) decreased to 42% of the normal, but the unbound nicotinic acid fell only to 93% of the normal.

In the skeletal muscle examined, similar but less pronounced changes occurred; they were not so clear-cut because the variation within the blacktongue group was greater, but there was a significant decrease both in the total nicotinic acid and in the coenzyme content. Again it appeared that only the bound fraction of the nicotinic acid decreased. Although the per-

centage decrease in the muscle was less than that in liver, considerably more nicotinic acid was lost from the musculature than from the liver owing to the difference between their weights. No change was observed in the kidney cortex.

Additional observations were made on dogs fed an excess of nicotinic acid, to determine the effect on the tissue content. Four dogs were maintained on the stock diet and given 20 mg. of nicotinic acid subcutaneously each day for 2 months. They were then decapitated and the tissues sampled for analysis. The results of these analyses are collected in table 1 and show no significant difference from the corresponding figures for normal dogs either in coenzyme or total nicotinic acid content. From this it appears that the dog is unable to store notable quantities of nicotinic acid or the pyridine nucleotides, and in this it resembles the rat (Axelrod, Madden and Elvehjem, '39; Dann and Handler, '41 b).

DISCUSSION

The findings reported above substantiate the conclusion of Kohn, Klein and Dann ('39) that there is a significant decrease from the normal in the coenzyme content of the liver and muscle of blacktongue dogs, although the decrease is not quite as great as they reported. Instead of a decrease in liver to 30% and in muscle to 65% of the normal, we now find decreases to 42% and 72% respectively. The figures given by Axelrod, Madden and Elvehjem ('39) also suggested such decreases, but the absolute values reported were almost twice as great as ours. Since there is not enough nicotinic acid present in the tissues to account for their high levels of coenzyme, it appears likely that their figures were too high. Such an error could arise either in the calibration of their yeast preparation or from incorrect evaluation of the purity of the standard diphosphopyridine nucleotide preparation with whose activity the tissues were compared. It has been pointed out by Handler and Dann ('41) that the latter is the more probable explanation. Recently, Axelrod, Spies and Elvehjem ('41) found a decrease from the normal in the coenzyme content of human

muscle in pellagra, corresponding to that found in canine blacktongue.

The suggestion has been made (Stannus, '40) that pellagra is due to impairment of the oxidizing systems of the cells, in turn due to decrease of the coenzyme level. The evidence of Kohn, Klein and Dann ('39) showed that this is unlikely, so far as known respiratory systems are concerned. Such a hypothesis takes no account of the tenacity with which the unbound nicotinic acid is retained in certain organs even when the coenzyme level falls; it is to be expected that nicotinic acid so retained has an equally vital function, although it is not possible to assign this function at present.

As it seems unlikely that the loss of coenzymes I and II from the body is the direct cause of the onset of pellagra or blacktongue and of death from these diseases, the question of the immediate cause of death remains unsolved. Observation of the experimental animals suggests that dehydration or the toxic reactions of the inflammation following the invasion of the alimentary mucous membranes may be the cause. Further experiments are now in progress to test the effect in acute blacktongue of (a) maintenance of body fluids by parenteral administration and (b) attack on the organisms invading the mucous membranes with chemo-therapeutic agents.

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THE MINIMUM REQUIREMENT OF RABBITS FOR dl-α-TOCOPHEROL

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TWO FIGURES

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Little is known concerning the nutritional requirements of various species for vitamin E. Since pure α-tocopherol, the most potent of the compounds having E activity, has become available the measurements of the need for this vitamin are now feasible. When sufficient data have been accumulated, perhaps certain generalizations will then be possible.

Evans and Emerson ('39) state that 3 mg. of α-tocopherol will allow a young mature female rat to bear a normal litter and that 0.75 mg. daily will protect the male rat against testicular degeneration (Evans, Emerson and Emerson, '39). Mackenzie and McCollum ('40) have reported that the rabbit needs no more than 1 mg. daily per kilogram of body weight. The present communication reports the results of experiments on the minimum quantities of dl-α-tocopheryl acetate needed to cure and maintain in good health rabbits suffering from nutritional muscular dystrophy caused by E-avitaminosis. Other factors, such as the method of administration, effect of sex and age will also be discussed. A preliminary report of this work has been published elsewhere (Eppstein and Morgulis, '40).

EXPERIMENTAL

Albino rabbits weighing 400 to 600 gm. were employed in these experiments. Weighings were made daily, and food and water given ad libitum. The progress of the dystrophy was

followed by growth curves and daily clinical observation. By these means an experienced observer can appraise the various stages of dystrophy as well as when the degree of creatinuria is included. This method has the advantage of requiring little time and, by dispensing with metabolism cages, it also permits the use of large numbers of animals for the tests. Biopsies on the gastrocnemius muscle were made in about half the cases as a further check on the degree of dystrophy. The microscopic examination and clinical findings were in good agreement. It should be emphasized that before cure was attempted the deficiency of E was maintained until the dystrophic condition became very definite as was determined by posture, sensitivity of muscles, stiffness, muscle tone, and by the ability of the animal to right itself from a supine position. This stage always occurred almost simultaneously with the downward break in the growth curve, the two occurring within 2 or 3 days of each other. Both criteria of the onset of dystrophy had to be fulfilled, however, before curative treatment was started. The expression "cure of muscle dystrophy," we believe, should only be used when dealing with animals showing active signs of the disease, and not merely suggestive symptoms. The interval of time during which a given supplement offered protection was determined by noting when the dystrophic symptoms reappeared.

Diet 113X had the following percentage composition: extracted wheat germ, 60; extracted casein, 15; extracted yeast, 5; sucrose, 7; salt mixture (McCollum's 185, modified by the addition of 45 gm. $CaCO_3$ to 80 gm. of the mixture), 3; lard, 8; and cod liver oil, 2%. The wheat germ was exhaustively extracted with hot, dry acetone. The casein and yeast were extracted seven times at room temperature by covering with petroleum ether (2 liters per kilogram of solids) for 12-hour periods. The lipid residues in these extracted materials were as follows: wheat germ about 2 mg. %; casein 50 mg. %; and yeast about 20 mg. %, or a total of 10–15 mg. of lipid from these sources per animal per day. For comparative purposes some animals were fed the Goettsch and Pappenheimer diet 13

(Goettsch and Pappenheimer, '31). Of the completely mixed diets (13 and 113X) enough was prepared at one time to last only 4–5 days. The supplies were kept in tightly-closed cans.

Appropriate amounts of dl-α-tocopheryl acetate [1] were dissolved in olive oil or in sesame oil and stored in brown bottles under CO_2 at 4°C. The dose of the supplement (always contained in 1 ml.) was given per os from a Mohr pipette calibrated with the oil solution. The rabbits, made dystrophic on either of the two diets, were fed graded doses of the ester ranging from 0.25 mg. to 6 mg. per day (calculated as the free tocopherol). The tocopherol was usually given daily but in some cases the appropriate multiple dose was fed at 2, 4, or 5 day intervals. Sometimes a 1 to 5 mg. dose was given and when the roughly calculated period of protection was almost up a second dose was fed, etc. The supplementation was continued for periods up to 45 days, and the length of time after its discontinuance until the first symptoms of dystrophy reappeared was noted. In a few instances the protection resulting from single massive doses of dl-α-tocopheryl acetate was studied. The minimum daily requirement for each animal was then calculated by dividing the total amount of the acetate fed (expressed as tocopherol) by the summation of the daily weights in kilograms from the time supplementation was begun until the animal showed signs of recurrent dystrophy. This method takes into account the variations in body weight and the results are expressed in milligrams per kilogram of body weight per day. Since it has been demonstrated that the racemic product is as effective as the naturally occurring tocopherol (Karrer, Fritzsche, Ringier and Salomon, '38), the results obtained are probably applicable to both forms.

RESULTS AND DISCUSSION

A tabulation of the data (table 1) shows a rather wide range of variation in requirement of α-tocopherol. Within reasonable limits, the magnitude of the individual dose fed is

[1] This product was generously supplied by Hoffman-La Roche, Inc., Nutley, New Jersey.

TABLE 1

α-tocopherol requirement.

RABBIT NO.	SEX	TOTAL AMOUNT OF α-TOCOPHEROL FED	PERIOD OF PROTECTION	α-TOCOPHEROL REQUIREMENT	RABBIT NO.	SEX	TOTAL AMOUNT OF α-TOCOPHEROL FED	PERIOD OF PROTECTION	α-TOCOPHEROL REQUIREMENT
		mg.	*days*	*mg./kg./day*			*mg.*	*days*	*mg./kg./day*
				Diet 113X					
598	M	40	114	0.16	678	F	7	15	0.30
599	M	40	114	0.16	625	F	13	26	0.31
543	M	5.8	23	0.19	629_1	M	20	32	0.31
654	M	10	26	0.19	659	M	8.1	22	0.32
634	F	3.2	16	0.20	588_1	M	38.4	60	0.34
650	M	10.6	25	0.23	628	M	25	44	0.34
595_1	F	36	72	0.23	658	F	9.4	23	0.35
595_2	F	23.5	36	0.23	629_2	M	26.5	31	0.36
630_1	F	22.5	53	0.23	663	M	10	18	0.36
588_2	M	27.5	48	0.24	600_1	F	40	58	0.37
637	F	14.8	36	0.24	672	F	5	8	0.37
594	M	42	87	0.25	676	M	7.5	17	0.37
602_1	F	42	73	0.26	601_2	M	23.8	32	0.39
602_2	F	30	40	0.26	635	F	15.5	34	0.40
603_2	M	30	42	0.26	661	F	12	23	0.40
593_2	M	21.2	32	0.27	593_3	M	45	61	0.41
589_1	F	45	75	0.28	601_1	M	40.8	65	0.43
603_1	M	42	73	0.29	662	M	26	40	0.44
620	M	5	13	0.29	664	F	20.3	39	0.44
589_2	F	29	35	0.30	585	M	38.4	59	0.45
600_2	F	24.1	35	0.30	671	F	5	9	0.49
614	M	33.6	54	0.30	590	M	42	53	0.53
630_2	F	26.3	42	0.30	586	F	61	75	0.60
				Diet 13					
520_2	F	22	38	0.17	511_1	F	111	72	0.57
502_2	M	44	74	0.19	502_1	M	117	86	0.58
506_2	F	16	26	0.23	524	F	106	67	0.59
518	M	57.5	88	0.23	525	M	90	59	0.59
511_2	F	68	85	0.23	512_1	M	126	76	0.60
511_3	F	25	20	0.38	501_1	M	120	87	0.68
512_2	M	81	67	0.38	517	M	204	91	0.70
549_2	M	35.5	37	0.39	508	M	208	105	0.83
501_2	M	72	59	0.43	515	F	135	68	0.89
506_2	F	60	42	0.45	503	F	120	64	0.96
513	M	78	63	0.50	520_1	F	222	85	1.03
549_1	M	40	48	0.50	506_1	F	184	79	1.07

The subscripts $_1$, $_2$, $_3$ refer to the first, second and third test performed on the same rabbit.

Diet 113X. Standard deviation, S = ± 0.095; mean = 0.32 ± 0.037.

Diet 13 (first twelve animals). Standard deviation, S = ± 0.12; mean = 0.34 ± 0.11.

apparently of no importance, but the data reveal a direct relationship between the total amount of tocopherol fed and the requirement in milligrams per kilogram of body weight per day. Thus the requirements on diet 13 range all the way from 0.17 mg./kg./day to 1.07 mg./kg./day, the lower values corresponding to the smaller total intake while the higher values are shown by rabbits receiving the much greater total amounts of α-tocopherol.

In many animals we failed to bring about a cure regardless of the amount of tocopherol fed. This was due to the fact that in some cases the tocopherol feeding was started too late or that too little tocopherol was given when the animals were already too seriously injured by severe E-avitaminosis. In some instances the animals were purposely fed subminimal quantities. Nevertheless, in nearly two-thirds of the 112 experiments successful cures were effected.

If the total amounts of tocopherol administered are plotted against the time during which the animals have been protected against dystrophy, one notes the following interesting things. In the case of the rabbits on diet 13 or diet 113X which received very small or only moderate supplements of tocopherol a good linear relationship is seen between the two variables; but in the case of rabbits on diet 13, which were fed massive amounts of tocopherol, this relationship is no longer linear. The demarcation is not sharp but the change from the linear to the non-linear correlation occurs when the total amount of tocopherol is in excess of 60 mg. In other words, as the quantity of α-tocopherol fed increases beyond a certain limit it becomes progressively less effective in the protection against dystrophy.

The inefficient utilization of α-tocopherol on the high levels of intake can best be explained by assuming that the animal tissues soon become saturated with the vitamin, the excess being excreted. Or, if a greater rate of destruction occurs, this takes place only after saturation of the tissues is complete. Such an interpretation is borne out, for instance, by the three tests performed on rabbit 511. This animal on a

total intake of 111 mg. (3 mg. per day) shows a minimum requirement of 0.57 mg./kg./day, but in another test with a total intake of 48 mg. (4 mg. per day) this value is only 0.23 mg./kg./day. Yet, when it was given a single massive dose of 25 mg. an intermediate degree of utilization of 0.38 mg./kg./day was obtained. Had the rate of destruction been greater on the higher levels, then it would be expected that the single

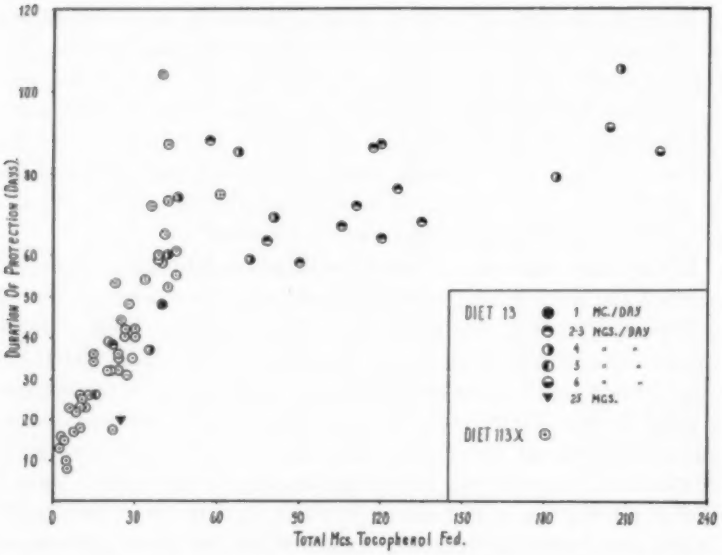

Fig. 1 Relation between the total amount of α-tocopherol fed and the duration of protection against dystrophy.

massive dose would be very inefficient; but if the tissues, depleted of their vitamin E reserves, could store a large portion of this dose allowing the excess to "spill over", in this instance, about 40%, the result could be easily explained. Successive tests carried out with other animals also bear out this interpretation. Assuming that the increased vitamin E requirement of the animals on a high total intake of tocopherol means that the tissues had become saturated with the vitamin,

the excess being eliminated or otherwise destroyed, then one can make a rough estimate of the ability of the animals to store the vitamin. This would be measured by the duration of the interval from the time supplementation was discontinued until dystrophic symptoms reappeared in these rabbits. Of course, there still remain two uncontrollable variables, namely, the relative mass of musculature of individual animals and the length of time during which only suboptimal reserves of the vitamin are available to the animal. Nevertheless, even a rough estimate of this "storage" capacity may be of some value. For the twelve rabbits on diet 13 with minimum requirements of tocopherol of 0.57 mg./kg./day or more this time-measure of storage capacity varies uniformly from 19 to 57 days, the average being 36 days. From these considerations it is obvious that a safe and convenient method of E supplementation is to feed at one time a calculated amount sufficient to last 1, 2 or even 3 weeks.

The values obtained on diet 13 when compared with those for diet 113X within the same general level of total supplementation are on the whole somewhat higher. By the methods of statistical analysis [2] the difference between the two means is not significant. Furthermore, the fact that animals on either diet become dystrophic in the same length of time, coupled with the remarkably good agreement between the lower requirements calculated on both diets, would indicate that these differences are not due to a greater amount of residual E in diet 113X but to the fact that few animals were used on diet 13 at the lower levels of total amounts of tocopherol fed.

In all probability several factors are responsible for the great range of variation in the minimum requirements, such as intestinal destruction of vitamin E, variations in the ability

[2] The formulae used as a test for significeane and for determining the probable error are those derived from the theory of fiducial limits. The interpretation of these values for the probable error is the same as for those obtained by the usual methods, but the concept of fiducial limits is theoretically sounder than that of the "probable error". For a brief discussion of this, see J. F. Kenney, Mathematics of Statistics, vol. II, pp. 177–186, 1939, D. Van Nostrand Co.

to absorb it, as well as the variable error in determining the period of protection. Leaving out of consideration the high values obtained on diet 13 where massive quantities were fed, one can see from the histograms (fig. 2) that most of the animals manifest an apparent need for between 0.2–0.4 mg. of dl-α-tocopherol per kilogram of body weight per day. The mean for diet 113X is 0.32, $\pm$0.037, and for the twelve animals on diet 13, excluding the high values associated with massive E intake, the average is 0.34 $\pm$ 0.11.

These values are considerably lower than the approximation of 1 mg./kg./day given by Mackenzie and McCollum ('40).

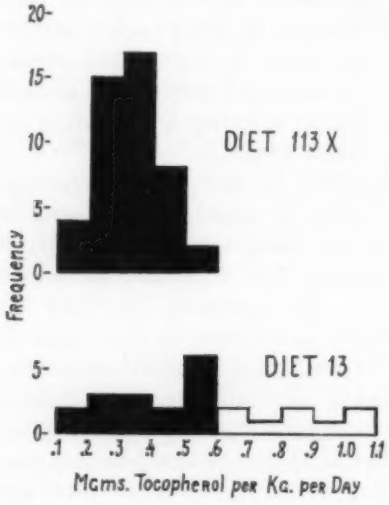

Fig. 2 Frequency distribution of tocopherol requirements in mg./kg. body weight/day. The unshaded portion represents the values when excessive amounts of vitamin were fed.

Parenteral use of tocopherol

To eliminate the variable factors of intestinal destruction and absorption, a number of experiments were made with intramuscular injections of dl-α-tocopheryl acetate. According to Knowlton, Hines and Brinkhous ('39) rats apparently

utilize the injected ester and it would seem reasonable to suppose that rabbits do also. Mattill ('40) reported that injections of the acetate ester were very inefficient in curing dystrophic rabbits. In our experience, too, twelve rabbits injected with from 5 to 10 mg. failed to be cured. Only a single animal, given one injection of 20 mg. in 1 ml. of olive oil, recovered.

Mattill ('40) suggested that possibly the ester to be utilized must first be hydrolyzed, the necessary enzyme being present only in the intestine. An alternative interpretation is that the oil is so poorly absorbed that the animals succumb before an adequate concentration of vitamin E in the tissues can be effected. To test this view four animals were injected with 5–10 mg. of tocopherol ester (in 1 ml. of oil) in four or five different places. The effect of such multiple injections should be the same as of a single injection of 20–25 mg. in 1 ml. of oil. A fifth animal was similarly treated with the free natural tocopherol.[3] All five animals so treated died. One rabbit treated with the ester apparently improved at first but then suffered a relapse. In order to clarify this problem experiments are now in progress with a water soluble tocopheryl ester.

Sex factor

No difference in tocopherol requirements could be detected between male and female rabbits, nor was there any difference in the per cent of cures effected in either sex. Mature animals of both sexes also showed the same vitamin E requirement.

Age factor

It was considered possible that the requirement for tocopherol would change with maturity. To test this, cured animals which had already become mature were made dystrophic again and retested, but no difference in E requirements due to age could be detected. If in the first period the animal had been given a large total amount, the E requirement for that period was, of course, higher. But whenever the total intake

[3] Kindly furnished by Merck and Co., Inc., Rahway, New Jersey.

of tocopherol was below approximately 60 mg., no significant change in E requirements could be found on comparing the immature animal with the mature.

SUMMARY

1. The minimum requirement of dl-α-tocopheryl acetate for rabbits as determined by the cure of dystrophy is probably about 0.32 mg. per kilogram of body weight per day (calculated as a free tocopherol).

2. The interval of time for which a supplement cured and protected the animal against dystrophy was directly proportional to the total amount of tocopherol fed when this amount was below approximately 60 mg. The reasons for this behavior are discussed.

3. The results of the present experiment suggest a convenient method for administering vitamin E supplements.

4. Sex or age do not affect the vitamin E requirements.

5. Intramuscular injections of dl-α-tocopheryl acetate failed to cure dystrophy.

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THE RIBOFLAVIN CONTENT OF FISH PRODUCTS

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The distribution of riboflavin in meat and meat products intended for human consumption has been reported by Darby and Day ('38), as determined by the rat growth method, and more extensively by Mickelsen, Waisman and Elvehjem ('39), as determined by the microbiological method. Hodson ('40) studied, by means of the fluorometric method, the influence of dietary riboflavin on the amount of this vitamin found in various tissues of chickens.

The riboflavin potency of livers from various domestic animals has been studied rather extensively. Daniel and Munsell ('37) and Hoagland and Snider ('30) determined biologically the riboflavin content of beef and pork liver. Additional data on the liver from the cow, calf, sheep, lamb and hog were reported by Saffry et al. ('40). Mickelsen et al. ('39) found that the riboflavin content of beef liver varied between 105 and 125 μg. per gram of dried material; veal liver between 100 and 135; pork liver between 80 and 90; and that one sample of lamb liver contained 90 μg. Hodson ('40) found that the liver of chickens fed a low riboflavin diet contained from 35 to 46 μg. of riboflavin per gram of dry tissue, while the liver of chickens fed a high riboflavin diet contained 94 to 129 μg. per gram of dry tissue. The results obtained by these investigators are in agreement, in that they have all shown that liver tissue is one of the most potent sources of riboflavin.

In view of the high riboflavin content of liver tissue obtained from domestic animals, it seemed of importance to determine the riboflavin content of fish livers and other by-products.

The riboflavin content of some of the more common poultry feedstuffs has recently been determined microbiologically by Culton and Bird ('41) and Billings and Biely ('40). On the whole, good agreement has been found between the biological and the microbiological values (Pratt et al., '40; Emmett et al., '41). The accuracy of the microbiological method per se has been determined for a large number of poultry feed samples by Billings and Biely ('40) in this laboratory and in co-operation with other laboratories.

MATERIALS AND METHODS

The results of the microbiological assay of fifteen liver meals, twelve by-product meals and ten fish meals for riboflavin are summarized in table 1. Livers from thirteen species of fish (samples 1 to 15, table 1) and several by-products were gathered during the autumn of 1940 and the winter of 1940–1941. The livers were collected into 40-pound tinned cans, sealed, and stored under refrigeration at an average temperature of −20°C.

Five-pound samples of the frozen livers were thawed and dried under vacuum at a temperature of 50–60°C. The greater portion of the liver oil was removed from the dried material by pressure using a Carver laboratory press. All the dried meals were analyzed for moisture and ether extract. The same procedure was followed with all samples of liver included in the study.

The viscera and roe meals, 16 to 23, were prepared in semi-commercial quantities (200 to 300 pounds) by drying in vacuo in a steam-jacketed dryer at 50–60°C. Meals from salmon milt and herring muscle, 24 and 26, were prepared in the laboratory, while meals from salmon heads, 25, were prepared in semi-commercial quantities.

"Stick water" meal was prepared by evaporating "stick water" under vacuum and drying it into a meal. "Stick

TABLE 1

The riboflavin content of fish products

SAMPLE NO.	SPECIES OF FISH	TISSUE	SOURCE	MOISTURE	FAT	RIBOFLAVIN CONTENT	
						Meal	Moisture and fat-free meal
				%	%	μg./gm.	μg./gm.
1	Red Cod	Liver	Laboratory	7.8	29.9	39.0	62.6
2	Red Cod	Liver	Laboratory	7.3	32.5	40.0	66.4
3	Ling Cod	Liver	Laboratory	4.1	23.9	48.0	66.6
4	Alaska Black Cod	Liver	Laboratory	16.5	16.7	60.0	89.8
5	Grey Cod	Liver	Laboratory	13.3	29.8	57.0	100.1
6	Chum Salmon	Liver	Laboratory	8.2	9.3	43.0	52.1
7	Pink Salmon	Liver	Laboratory	29.2	13.1	56.0	97.4
8	Sockeye Salmon	Liver	Laboratory	29.6	22.5	38.0	79.3
9	White Spring Salmon	Liver	Laboratory	8.0	21.4	62.5	88.5
10	Soupfin Shark	Liver	Laboratory	15.4	18.3	64.0	96.5
11	Dog Fish	Liver	Laboratory	11.5	31.6	48.0	84.3
12	Halibut	Liver	Laboratory	17.7	18.9	44.0	69.4
13	Albacore Tuna	Liver	Laboratory	8.2	30.7	62.0	101.4
14	Herring	Liver	Laboratory	8.5	16.4	67.0	89.2
15	Sockeye Salmon	Liver	Laboratory	9.8	17.1	67.5	92.3
16	Alaska Black Cod	Viscera [1]	Laboratory	15.5	26.3	30.5	52.4
17	Chum Salmon	Viscera [1]	Semicommercial	12.4	9.7	33.0	42.3
18	Pink Salmon	Viscera [1]	Semicommercial	8.7	13.1	33.5	42.8
19	Sockeye Salmon	Viscera [1]	Semicommercial	12.5	14.8	40.5	55.7
20	Salmon	Roe	Semicommercial	10.3	4.8	22.7	26.7
21	Salmon (heat treated)	Roe	Semicommercial	12.5	18.2	24.5	35.3
22	Salmon (raw)	Roe	Semicommercial	13.2	11.6	35.7	47.4
23	Salmon	Roe	Semicommercial	8.9	8.8	34.2	41.5
24	White Spring Salmon	Milt	Laboratory	9.0	3.9	10.0	11.4
25	Salmon	Heads	Semicommercial	9.7	20.5	15.0	21.4
26	Herring	Muscle	Laboratory	3.5	23.7	10.0	13.7
27	"Stick meal"	Stick	Semicommercial	3.1	22.1	25.0	33.4
28	Pilchard	Meal	Commercial	....	....	13.2	
29	Pilchard	Meal	Commercial	....	....	11.8	
30	Pilchard (oily)	Meal	Commercial	....	....	13.6	
31	Salmon	Meal	Commercial	....	....	9.0	
32	Herring	Meal	Commercial	....	....	9.2	
33	Herring (oily)	Meal	Commercial	....	....	11.0	
34	Herring (vacuum dried)	Meal	Commercial	....	....	16.0	
35	Herring (Japanese) sun-dried	Scrap	Commercial	....	....	18.0	
36	Herring (Japanese) sun-dried	Scrap	Commercial	....	....	22.0	
37	Herring (Japanese) sun-dried	Scrap	Commercial	....	....	22.0	

[1] In the process of cleaning salmon probably two-thirds of the liver stays with the head, since part of the liver is in that portion of the stomach cavity which is cut off with the head. The pink and chum viscera meal contains that portion of the liver which remained in the body cavity, but roe was removed. The sockeye viscera meal was prepared from material, however, from which both roe and all remaining liver was removed.

water'' is ordinarily a waste product of the wet process of producing fish meal (Beall, '33). It is the watery solution remaining after separation of oil from the liquid pressed from cooked fish. It contains much of the water-soluble vitamins present in the original fish (Lassen, '40).

Samples 28 to 35 are typical of fish meals manufactured and used in British Columbia. Samples 35 to 37 (herring scrap) represent a Japanese product ordinarily known to the trade as ''sun-dried herring scrap.''

Representative samples of each of the meals were thoroughly ground and stored in glass bottles in a dark room. The procedure followed in the microbiological tests was similar to that described by Snell and Strong ('39) and Billings and Biely ('40). Each sample of meal was tested at several levels, and appropriate controls (positive and negative) were used in each instance. In addition to the microbiological test, one sample of liver meal, viscera meal and roe meal was tested biologically on chicks. Close agreement was obtained between the biological and microbiological tests (Pratt, Biely and Fisher, '40).

DISCUSSION

It will be seen from the data presented in table 1 that with one or two exceptions the riboflavin content of the fish liver meals is uniformly high. The meals prepared from the fish viscera were found to rank second to fish livers in riboflavin content. Fish muscle meal, as is the case with the muscle of domestic animals, was found to be low in riboflavin as compared with liver meal. It is not without interest to note that while fish roe has been found to contain appreciable amounts of riboflavin, herring milt is definitely low. ''Stick water'' meal was found to contain appreciable amounts of riboflavin. While it is thus a fairly valuable source of riboflavin, it should be remembered that ''stick water'' meal protein has a low biological value (Pratt, Biely and Fisher, '40).

Fish meals, as will be seen from table 1, vary considerably in riboflavin content. Wilgus et al. ('35) have shown that the

riboflavin content of fish meals may be affected by the method of manufacture and by the kinds of material used. Since liver meal and viscera meal are relatively high in riboflavin, the amount of these products included in fish meals would affect their riboflavin content.

The results of the microbiological determinations show that of the various fish products assayed for riboflavin, liver tissue is one of the most potent sources of this vitamin. It may be of interest to note that the distribution and relative content of riboflavin in the various tissues of sea fish are similar to those found in corresponding tissues of domestic animals. Our results are in agreement with those obtained by Lunde in Norway ('39), who found that fish liver and roe are especially rich in riboflavin.

SUMMARY

The riboflavin content of fish products has been determined according to the microbiological method of Snell and Strong.

Of the materials examined, fish liver meal proved to be richest in riboflavin content; viscera, roe and "stick water" meal were next in value; muscle meal and milt meal were lowest.

Fish meals showed considerable variation in their riboflavin content. This may be attributed to different methods of manufacture and various types of material used.

Fish liver meal, like liver meal prepared from domestic animals, is one of the richest natural sources of riboflavin.

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THE EFFECT OF AUTOCLAVING ON THE NUTRITIVE VALUE OF THE PROTEINS IN COTTONSEED MEAL[1]

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TWO FIGURES

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The literature describing the effect of heat on the food value of proteins has been summarized by Hayward, Steenbock and Bohstedt ('36), Waisman and Elvehjem ('38), and Greaves, Morgan and Loveen ('38). Casein, edestin, and meat proteins are adversely affected while soy and other bean proteins are improved by such treatment. Osborne and Mendel ('17) and Gallup ('28) reported that excessive steaming or autoclaving of cottonseeds or commercial cottonseed meals damaged the protein. However, interpretation of their data is complicated by the presence of the toxic substance, gossypol, or derivatives resulting from it by various treatments. The problem is sufficiently important to warrant further investigation inasmuch as most of the 2,000,000 tons of cottonseed cake and meal produced annually in this country is subjected to a cooking procedure prior to the pressing operation. It is then used as a source of protein in stock feeds. Information relating to the effect of cooking on the nutritive value of the proteins might be helpful in modifying the procedure to improve the

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food value of cottonseed meals. In the present study, gossypol-free cottonseed meats, prepared by ethyl ether extraction, were used to determine the effect of autoclaving on the nutritive value of the proteins.

EXPERIMENTAL

Whole cottonseed meats [4] were ground to pass a 20-mesh screen and extracted with ethyl ether in a large scale Soxhlet-type extractor capable of holding 15 pounds of meal in one charge. After drying, the oil- and gossypol-free meats were re-ground (burr-mill) to pass a 60-mesh screen. Portions were treated in a steam-jacketed autoclave by spreading in thin layers ($\frac{1}{2}$–1 cm.) on shallow trays. The temperature of the steam was measured by a thermometer set into an outlet through which a slight flow of steam was allowed to escape. The temperature of autoclaving remained constant at 123–124°C. (17 pounds) and the period of exposure to such treatment was measured from the time the inner chamber had reached 120°C.

The various cottonseed preparations were evaluated by their ability to stimulate growth in ad libitum feeding. Male rats (Sprague-Dawley) at weaning were placed in individual screen-bottom cages. They were weighed thrice weekly and food consumption data were recorded. The equicaloric diets had the compositions shown in table 1, with the vitamin-B complex furnished by liver extract and thiamine chloride as suggested by Elvehjem.[5]

The results of feeding the meal autoclaved for different periods of time and at 12 and 24% protein levels are presented in figure 1 and table 2. The conclusions have been based upon the results obtained at the 12% protein level. The groups of rats fed the meals at the 24% protein level, although few in number, have been included because they furnished a good check upon the other results, and because the better rate of growth, characteristic of experience with varying amounts of

[4] Courtesy of W. H. Jasspon, Perkins Oil Co., Memphis, Tennessee.

[5] Private communication from C. A. Elvehjem, University of Wisconsin.

other proteins (reviewed by Hamilton, '39), showed that no traces of gossypol or other toxic substances remained in the meal.

The nutritive value of the cottonseed meal decreased during autoclaving until, after 2 hours of such treatment, it was capable only of maintaining weight when fed at the 12% protein level. Although the varied conditions used by different investigators prevent direct comparison, the cottonseed

TABLE 1

Composition of diets

Ethyl ether-extracted cottonseed meats	24.8 [1]	49.6 [1]
Hydrogenated cottonseed oil [2]	19.5	19.0
Sucrose	51.7	27.4
Salts [3]	2	2
Liver extract [4]	2	2
Haliver oil [5]	20 mg. (1 drop) per 100 gm.	
Protein content (N × 6.18)	12.7	25.0
In addition, each animal received 50 μg. of thiamine chloride in solution by mouth thrice weekly.		

[1] Approximate composition also of the diets containing extracted meats autoclaved for ½, 1, and 2 hours.

[2] Crisco. The amount used was calculated to bring the total lipid concentration to 20%, approximately.

[3] Hubbell, Mendel and Wakeman ('37) mixture no. 351.

[4] Liver concentrate, 1:20. Contains nitrogen equivalent to 50% protein. Purchased from the Wilson Laboratories Co.

[5] According to manufacturer's assay, contained 50,000 vitamin A and 10,000 vitamin D units (U.S.P. XI) per gram.

proteins appear to be somewhat more sensitive to heat damage than other proteins. Thus, Waisman and Elvehjem ('38) autoclaved edestin at 120°C. for 5 hours and found that the rat growth increment (one pair) decreased from 105 to 25 gm. (18% protein, 6 weeks growth period). Seegers and Mattill ('35) heated dried beef liver at 120° for 72 hours and found a marked decrease in protein value. Greaves, Morgan and Loveen ('38) reported that the nutritive value of casein, expressed in terms of grams of gain per gram of protein eaten, declined from 2.4 to 1.4 after heating the casein 24 hours in an

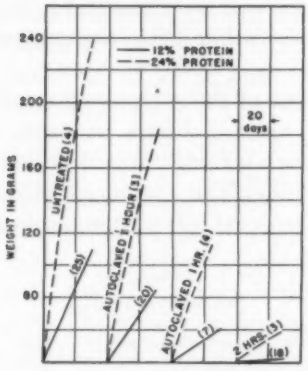

Fig. 1 Male rat growth rates obtained with diets containing ether-extracted cottonseed meats autoclaved at 17 pounds pressure for different lengths of time. The figures in parentheses indicate the number of animals for which the curves are an average.

oven at 130° (12% protein level). Mitchell, Burroughs and Beadles ('36) observed a slight decrease in biological value when peanuts were roasted at 204–232° for 30 to 35 minutes. The difference between the effect of wet heat and dry heat on the proteins obviously requires further investigation. In the case of soy bean proteins tested at an 18% level, autoclaving for 1½ hours at 125° increased the nutritive value from

TABLE 2

The effect of autoclaving on the nutritive value of the proteins in ether-extracted cottonseed in ad libitum feeding.

TREATMENT OF MEAL	PROTEIN CONTENT OF DIET	NO. RATS	DAYS	GROWTH GAINS		AVERAGE FOOD CONSUMPTION	GM. OF GAIN PER GM. OF PROTEIN CONSUMED
				Range	Average		
	%			gm.	gm.	gm.	
None	12.7	7	31	66–72	68.0	268	1.99
Autoclaved ½ hr.	12.5	8	31	42–54	45.0	227	1.58
Autoclaved 1 hr.	12.5	4	31	13–20	17.5	137	1.08
Autoclaved 2 hr.	12.9	3	31	2– 4	3.3	100	0.28
None	25.0	3	33	174–179	176.7	383	1.84
Autoclaved ½ hr.	24.95	3	31	108–175	142.7	311	1.84
Autoclaved 1 hr.	24.7	4	31	82– 92	84.0	260	1.31
Autoclaved 2 hr.	24.7	3	35	19– 30	23.0	179	0.52

0.3 to 1.2 gm. of gain per gram protein eaten, whereas dry heat was much less effective (Hayward, Steenbock and Bohstedt, '36).

AMINO ACID SUPPLEMENTATION STUDIES

In an attempt to determine which amino acids were destroyed or rendered biologically unavailable by the steam treatment, experiments were undertaken in which crystalline amino acids were used to supplement diets containing the 2-hour autoclaved ether-extracted meal fed at a 12% protein level. During a 15-day preliminary period, five rats were given the basal diet (A), five were given diet A in which 1% glycine replaced 1% sugar (B), and five were given diet A in which 2% glycine replaced 2% sugar (C). Glycine was used in order to ascertain the effect of adding a non-essential amino acid. No growth differences were detectable. One rat from each group was then placed upon a basal diet in which a portion of the sugar was replaced by supplements corresponding to 2% lysine (D), 1% lysine (E), 1% lysine and 1% histidine (F), 1% histidine (G), and 2% histidine (H). During the succeeding 15 days, the weight changes shown in figure 2 were observed. The animals receiving lysine gained an average of 11.5 gm., whereas those given histidine gained only 5.3 gm. Inasmuch as the lysine plus histidine inclusion produced no greater response than did lysine alone, it was concluded that the effect of histidine was negligible. This was confirmed in a subsequent 16-day experiment in which the growth of three rats given the basal diet plus histidine (1%) was equal to that of three others receiving glycine (1%).

The responses given by the two groups receiving 1 and 2% lysine indicated that the lower level was sufficient to overcome the lack of this amino acid in the autoclaved meal.

At the end of the experimental periods, several of the animals were transferred to diets containing untreated cottonseed meal. They immediately began to grow at rates of 3 to 4 gm. a day indicating that the metabolic responses to the proper amino acid combination had not been impaired.

Waisman and Elvehjem ('38) reported that lysine was the limiting factor in autoclaved edestin and Greaves, Morgan and Loveen ('38) concluded that lysine was the first amino acid damaged in heated casein, and histidine the second. Harris and Mattill ('40) showed that a loss in free amino nitrogen paralleled a loss in in vitro digestibility of meat proteins after

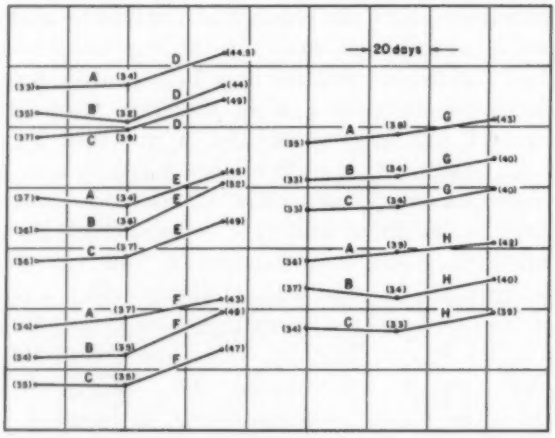

Fig. 2 Rat weight changes observed with amino acid supplementation of the ration containing ether extracted cottonseed meats autoclaved for 2 hours (12% protein level). A, no supplement; B, 1% glycine; C, 2% glycine; D, 2% lysine (3% lysine dihydrochloride); E, 1% lysine (1.5% lysine dihydrochloride); F, 1% lysine and 1% histidine (1.5% lysine dihydrochloride and 1.25% histidine hydrochloride); G, 1% histidine (1.25% histidine hydrochloride); and H, 2% histidine (2.5% histidine hydrochloride). In each case the added compounds replaced an equivalent amount of sugar. The figures in parentheses represent the rat weights in grams.

hot alcohol extraction. In the present instance, the lack of marked response to lysine and histidine would appear to indicate that either these amino acids were available in adequate or almost adequate amounts in the autoclaved cottonseed meal, or the unavailability of one or more other amino acids was sufficiently critical to mask the benefit of the addition of lysine or histidine.

SUMMARY

As measured in ad libitum growth experiment with rats, steam autoclaving (17 pounds pressure) markedly reduced the nutritive value of the proteins in gossypol-free (ether-extracted) cottonseed meats. The 2 hour-autoclaved meal when present in quantity sufficient to give 12% protein in the diet permitted a gain in grams per gram protein eaten of 0.52 compared with 2.0 for the unautoclaved meal. Animals fed the 2 hour-autoclaved meal were only slightly benefitted by supplementation with lysine. Supplementation with histidine was ineffective.

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